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Retro peptide-hybrids as selective inhibitors of the Dengue virus NS2B-NS3 protease

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ABSTRACT

New chemotherapeutics against Dengue virus and related flaviviruses are of growing interest in antiviral drug discovery. The viral serine protease NS2B-NS3 is a promising target for the development of such agents. Drug-like inhibitors of this protease with high affinity to the target are not available at the moment. The present work describes the discovery of new retro di- and tripeptide hybrids that do not necessarily require an electrophilic "warhead" to achieve affinities in the low micromolar range. The most active sequence in this series is the tripeptide R-Arg-Lys-Nle-NH_2. By variation of the *N*-terminal groups (R) it could be shown that the previously described arylcyanoacrylamide moiety is a preferable group in this position. Retro tripeptide hybrids were found to be more active and more selective than retro dipeptide hybrids. A significant selectivity towards the Dengue virus protease could be shown in a counterscreen with thrombin and the West Nile virus protease. Alternative sequences to R-Arg-Lys-Nle-NH_2 did not have higher affinities towards the Dengue virus protease, similar to retro-inverse sequences with D-lysine and D-arginine residues. The results of a competition assay with the known inhibitor aprotinin indicate that the *N*-terminal arylcyanoacrylamide residue of this compound class binds near the catalytic center of the enzyme.

1. Introduction

Antiviral drugs for the treatment of Dengue virus infections are of growing interest in medicinal chemistry. On the one hand, there is a problem of periodic, epidemic outbreaks with millions of infected people in Latin America, Africa (Amarasinghe et al., 2011), South and Southeast Asia and Oceania. On the other hand an advance of the transmitting vectors towards the northern hemisphere can be observed. Because the Dengue virus is not transmittable without a vector, the reason for its expansion is based on the spread of the habitat of the *Aedes* (*Stegomyia*) mosquitoes, probably caused by global warming. Mosquito eggs of the genus *Stegomyia albopicta* were found in Germany in 2007, which probably stem from adults transported by motor vehicles over long distances (Pluskota et al., 2008). In September 2010 the first two cases of autochthonous Dengue fever occurred in metropolitan France (La Ruche et al., 2010). Transmissions of the Dengue virus with several infections could be verified in Texas, Hawai and, most recently, in Florida (Franco et al., 2010). Against this background there is an urgent need for a chemotherapeutic agent or a vaccine against Dengue virus, but neither is available at the moment.

Flaviviruses such as the Dengue virus (DEN) have a single strand RNA genome, which codes for a polyprotein that contains structural and non-structural (NS) proteins. The NS3 serine protease with its cofactor NS2B is essential for the posttranslational processing of the viral polyprotein and consequently a critical parameter for the viral life cycle and replication mechanism. Therefore an inhibition of this protease is an interesting approach for the development of new pharmaceuticals against diseases caused by the Dengue virus and other flaviviruses (Lescar et al., 2008). Up to now, small-molecule, non-peptidic inhibitors have not reached sufficient affinities against the DEN protease. Therefore, at the moment, peptide-based compounds represent a promising alternative for the development of selective and potent inhibitors.

It could be shown that a dibasic sequence (primarily with two arginine residues) of a peptide substrate in the P_1 and P_2 positions next to the cleavage site is essential for high affinity against the DEN NS2B-NS3 protease (Yusof et al., 2000). By further investigations Li et al. (2005) found that a lysine in P_3 and a norleucine in P_4 position are preferred, so that consequently the sequence Nle-Lys-Arg-Arg is a promising lead for the development of small peptidic inhibitors. Yin et al. (2006a) combined this and alternative *N*-terminal benzoyl capped sequences with different "warhead" electrophiles at the *C*-terminus to establish an additional covalent binding to the catalytic serine, resulting in increased affinity. By additional analysis of various *N*-benzoyl capped di-, tri- and predominantly tetrapeptides, containing an aldehyde electrophile, Yin et al. (2006b) presented compounds with activities in the lower micromolar range. Different *N*-terminal "caps" of tripeptide aldehydes

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without norleucine (R-Lys-Arg-Arg-H and R-Lys-Lys-Arg-H) were analyzed by Schüller et al. (2011) at DEN and the closely related West Nile virus (WNV) protease. They generally found a preference of all explored inhibitors towards the WNV target, independent of the used caps or peptide sequences.

Recently we published two different approaches for small-molecular inhibitors containing a cinnamyl pharmacophore, which probably bind near the catalytic center of the protease. In the first work α-ketoamides were evaluated as possible covalent modifiers for the DEN protease with antiviral activity in cell-culture experiments, where cinnamyl containing structures gave best activity results (Steuer et al., 2011). The second work described the arylcyanoacrylamide scaffold as an alternative structural feature to target the DEN and WNV protease, with satisfying selectivity against thrombin (Nitsche et al., 2011). In both works the cinnamyl double bond turned out to be an indispensable structural feature for good activity. Because the affinity of these congeners is not sufficient for advanced pharmacological studies, we decided to expand the inhibitors towards additional recognition pockets of the DEN protease. For this we added small retro peptide sequences at the aromatic system (predominantly the arylcyanoacrylamide moiety), resulting in the organo-peptide hybrids reported here. These compounds are the first reported Dengue-selective inhibitors with a retro peptide sequence and an alternative structural component for one of the two obligatory arginine residues. In the present work we also show that there is no absolute necessity for a highly electrophilic "warhead" like an aldehyde function to obtain high affinities. In comparison to the more complex synthesis of peptidyl aldehydes, our compounds are synthetically easily accessible, and less prone to metabolic inactivation or off-target effects that are elicited by binding towards other biological nucleophiles.

2. Materials and methods

2.1. Chemical synthesis

2.1.1. Chemical reagents

The precursor acids as *N*-terminal caps were obtained directly or synthesized (Section 2.1.2.) from commercially available chemicals (Sigma–Aldrich, Germany). The protected amino acids were purchased from Orpegen (Germany), Novabiochem, Merck (Germany) and Sigma–Aldrich (Germany). HBTU was obtained from Iris Biotech (Germany). The Rink amide resin was purchased from Rapp Polymere (Germany) and Novabiochem, Merck (Germany) and was of an average capacity of 0.65 mmol/g. All other chemicals or solvents were obtained from Sigma–Aldrich (Germany) and were of analytical grade.

2.1.2. Synthesis of precursor cap molecules

The *N*-terminal caps 4-[(*E*)-3-amino-2-cyano-3-oxoprop-1-en-1-yl]benzoic acid, 4-[(*E*)-2-cyano-3-(cyclopropylamino)-3-oxoprop-1-en-1-yl]benzoic acid, 3-[(*E*)-2-cyano-3-(cyclopropylamino)-3-oxoprop-1-en-1-yl]benzoic acid and (*E*)-2-cyano-3-[4-hydroxyphenyl]acrylic acid were synthesized by aldol condensation as described by Nitsche et al. (2011). The 4-[(*E*)-3-amino-3-oxoprop-1-en-1-yl]benzoic acid cap for compound **14** was synthesized via Heck reaction from 4-iodobenzoic acid and acrylamide (Fig. 1) as described by Zhao et al. (2002). (*E*)-2-Oxo-4-phenylbut-3-enoic acid as a precursor for compound **19** was synthesized according to Steuer et al. (2011).

2.1.3. Synthesis of capped peptides

The peptides with different caps were synthesized by solid phase peptide synthesis using the Fmoc protocol. Rink amide resin was left to swell for at least 30 min with CH_2Cl_2 in a disposable syringe, washed five times with NMP and treated three times (for 2 min) with a piperidine solution (20% in NMP) to cleave the Fmoc group. After repeated washing with NMP (5×) the protected amino acid was coupled. For each coupling step, a concentrated solution of HBTU (5 equivalents in NMP, 1.5 ml per 100 mg resin) was added to the protected amino acid (5 equivalents). Immediately before coupling, a DIPEA solution (20% in NMP, 0.5 ml per 100 mg resin) was added. The coupling step was performed for 45 min, then the resin was washed again five times with NMP, the Fmoc group was cleaved as described before and the whole coupling procedure was repeated for the next amino acid. After the last cleavage of the Fmoc group the cap acid was coupled in an analogous procedure, before the resin was finally washed five times with NMP and CH_2Cl_2 and dried in vacuum. After drying, the resin was treated with the cleavage solution (95% TFA, 2.5% water, 2.5% TIPS, 3 ml per 100 mg resin) for 2 h before the peptide was precipitated by pouring the cleavage solution into cold diethyl ether (25 ml per 100 mg resin). The cleavage procedure was repeated two times for 30 min. After centrifugation and washing with diethyl ether the residue was dried in vacuum and purified by preparative HPLC on an ÄKTA Purifier, GE Healthcare (Germany), with a RP-18 pre- and main column (Reprospher, Dr. Maisch GmbH, Germany, C18-DE, 5 μm, 30 × 16 mm and 120×16 mm). The conditions were: eluent A: water (0.1% TFA), eluent B: methanol (0.1% TFA), flow rate: 8 ml/min, gradient: 10% B (2.5 min), 100% B (23.5 min), 100% B (26 min), 10% B (26 min), 10% B (30 min). Detection was performed with a UV-detector at 214, 254 and 280 nm. The peptides were obtained as solid powders after lyophilization and characterized by HR-ESI mass spectrometry on a Bruker microTOF-Q II instrument. Purity was determined by HPLC on an Agilent 1200 HPLC system with a DAD detector and an alternative LC–MS system using an Agilent 1200 HPLC system with a multiple-wavelength detector combined with the Bruker microTOF-Q II instrument on a RP-18 column (ReproSil-Pur-ODS, Dr. Maisch GmbH, Germany, 3.3 μm, 50 × 2 mm). All evaluated compounds were obtained with a purity of at least 95%.

2.2. In vitro assays

2.2.1. DEN and WNV protease expression and purification

The DEN (serotype 2) and WNV NS2B-NS3 proteases were expressed and purified according to the protocol described by Steuer et al. (2011). For both proteases the cofactor is covalently connected to the protease domain by a glycine-serine linker (GGGGSGGGG).

2.2.2. Substrate synthesis

The internally quenched DEN NS2B-NS3 protease substrate Abz-Nle-Lys-Arg-Arg-Ser-3-(NO_2)Tyr and the WNV substrate Abz-Gly-Leu-Lys-Arg-Gly-Gly-3-(NO_2)Tyr were synthesized by solid-phase synthesis on Rink amide resin according to the Fmoc-protocol as described before for the inhibitor peptides (Section 2.1.3.). They were also purified by preparative HPLC and the purity (>95%) was assessed by HPLC-UV with the instruments and conditions described before. The identity was confirmed by MALDI-TOF-MS on a Bruker BIFLEX III instrument.

2.2.3. DEN and WNV protease assay

The DEN and WNV protease assays were performed as described previously (Steuer et al., 2009). In short, continuous enzymatic assays were performed on a BMG Labtech Fluostar OPTIMA microtiter fluorescence plate reader using black 96 well V-bottom plates from Greiner Bio-One (Germany). The excitation wavelength was 320 nm and the emission was monitored at 405 nm. The inhibitor concentration was 50 μM. The inhibitors were preincubated for 15 min with the DEN protease (100 nM) or WNV protease

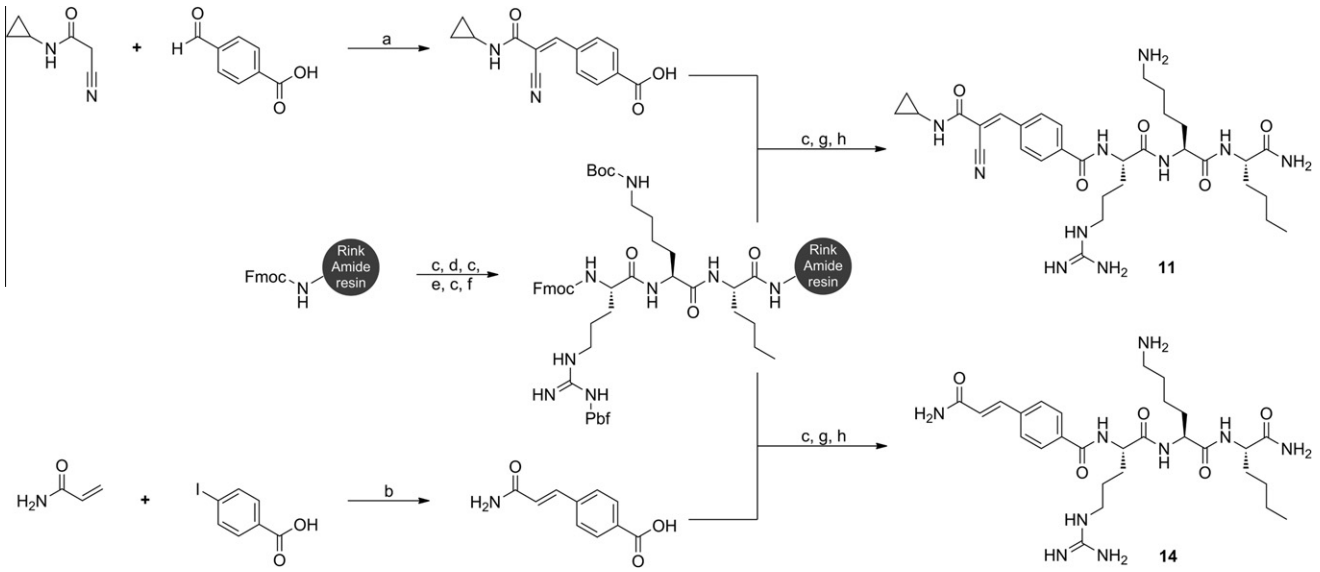


Fig. 1. Synthetic scheme of compounds **11** and **14** as examples for two cap groups combined with the same tripeptide sequence. (a) *N*-methylpiperazine, MeOH; (b) K_2CO_3, Bu_4NBr, $Pd(OAc)_2$, H_2O; (c) piperdine, NMP; (d) Fmoc-Nle-OH, HBTU, DIPEA, NMP; (e) Fmoc-Lys(Boc)-OH, HBTU, DIPEA, NMP; (f) Fmoc-Arg(Pbf)-OH, HBTU, DIPEA, NMP; (g) HBTU, DIPEA, NMP; (h) TFA, TIPS, H_2O.

(150 nM), respectively. Afterwards, the reaction was initiated by the addition of the substrate to a final concentration of 50 μM. The activity of the enzyme was determined as the slope per second (RFU/s) and monitored for 15 min. Determinations were performed in triplicate and the experimental values were averaged. K_i-values were determined by duplicate experiments with eight different substrate concentrations at three different inhibitor concentrations and without inhibitor. The calculation was done using Prism 5.0 (GraphPad Software, Inc.).

2.2.4. Thrombin assay

The thrombin assay was performed as a continuous fluorimetric assay on a BMG Labtech Fluostar OPTIMA microtiter fluorescence plate reader using black 96 well V-bottom plates from Greiner Bio-One (Germany). The excitation wavelength was 355 nm and the emission wavelength was 460 nm. The protease was assayed using the substrate Boc-Val-Pro-Arg-AMC, purchased from Bachem (Germany). The final concentrations of the enzyme and substrate were 10 nM and 50 μM, respectively. The inhibitors were preincubated with the enzyme for 15 min at a concentration of 25 μM. The cleavage reaction was initiated by addition of the substrate. The assay buffer consisted of 50 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.05% Tween 20 (Diamond, 2011). The activity of the enzyme was determined as the slope per second (RFU/s) and monitored for 10 min. K_i-values were determined as described for the DEN and WNV proteases (Section 2.2.3.).

2.2.5. Tryptophan quenching assay

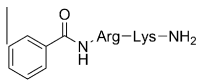
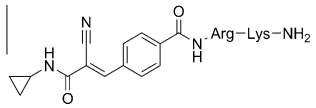
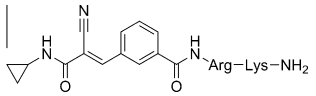
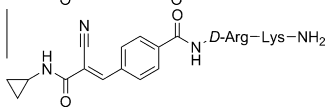
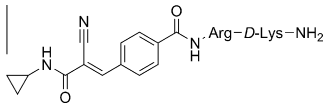
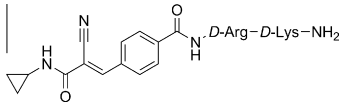
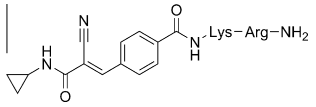
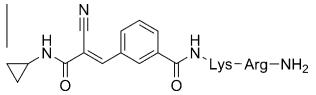
This assay was performed as described by Bodenreider et al. (2009). In short, the competition assay was performed with six different inhibitor concentrations (0, 5, 10, 20, 25 and 50 μM) and the DEN protease (4 μM) using the assay system described by Steuer et al. (2009). Additionally, the inhibitor (50 μM) was incubated with aprotinin (10 μM). As negative control, the autofluorescence of DEN protease and aprotinin without inhibitor at the concentrations mentioned above was determined. The autofluorescence of the samples was monitored on a Tecan Safire II instrument with an excitation wavelength of 280 nm and an emission wavelength of 330 nm. All determinations were performed in duplicate and averaged.

3. Results and discussion

3.1. Retro dipeptides

Retro and retro-inverse peptide sequences of target substrates are often used for new investigations in protein inhibition and for a deeper understanding of the intermolecular recognition (Chorev and Goodman, 1995). At retro peptides only the *C*- and *N*-termini of a given peptide sequence are interchanged, whereas in retro-inverse peptides the stereocenters are inverted as well. We investigated different retro, retro-inverse, semi retro-inverse and non-retro dipeptides, which are shown in Table 1, containing arginine, lysine and a benzoyl or a *meta*- or *para*-connected arylcyanoacrylamide moiety as *N*-terminal cap, which appears to be a suitable replacement for the arginine residue in P_1 position. Regarding the retro-peptides **1**, **2** and **3** we found the best activities at DEN and WNV proteases of the *meta*-connected arylcyanoacrylamide capped derivative **3**, directly followed by the *para*-connected compound **2**, with K_i-values between 15 and 30 μM. The benzoyl capped compound **1** shows lower activity at DEN (K_i = 39.6 μM) and WNV protease (K_i = 102 μM). This indicates that the arylcyanoacrylamide scaffold (cpd. **2** und **3**) is a more preferred moiety for molecular interactions with the DEN and WNV proteases (Nitsche et al., 2011). An inversion of the sequence from R-Arg-Lys-NH_2 to the non-retro sequence R-Lys-Arg-NH_2 in compounds **7** and **8** leads to loss of activity at the DEN and nearly invariant activity at the WNV protease, with a tendency of lower activity at the *meta*-connected derivative **8** with a K_i-value of 33.8 μM. This is in agreement with the conclusion that the WNV protease favors lysine and the DEN protease arginine residues in P_2 position (Schüller et al., 2011; Stoermer et al., 2008) and underlines again the notion that the cap group acts as an arginine replacement in P_1. In summary, the exchange of the arginine residue by a lysine and vice versa (cpds. **2** vs. **7** and **3** vs. **8**) leads to a complete loss of activity at DEN protease but has no influence on the activity at WNV protease. The DEN protease is apparently more sensitive to molecular changes in the retro-peptide inhibitors. The semi retro-inverse peptides **4** and **5** and the fully retro-inverse derivative **6** show only low activities at both viral targets. This demonstrates that retro peptides are an excellent choice for the

Table 1
Dipeptides with different lysine and arginine sequences.

No.	Structure	Type[f]	DEN	WNV	Thrombin
1		*r*	(41.0 ± 2.3)%[a] K_i = 39.6 ± 3.8 μM[d]	(28.4 ± 0.8)%[b] K_i = 102 ± 17.7 μM[d]	(8.6 ± 5.5)%[c]
2		*r*	(63.8 ± 4.3)%[a] K_i = 29.0 ± 5.5 μM[e]	(60.4 ± 4.7)%[b] K_i = 26.4 ± 3.7 μM[d]	(19.5 ± 5.2)%[c] K_i > 100 μM[e]
3		*r*	(68.6 ± 5.6)%[a] K_i = 15.4 ± 1.8 μM[d]	(67.9 ± 7.7)%[b] K_i = 24.9 ± 1.8 μM[d]	(10.7 ± 0.4)%[c]
4		*sri*	(18.2 ± 4.2)%[a]	(24.7 ± 4.1)%[b]	(6.9 ± 5.8)%[c]
5		*sri*	(15.5 ± 1.5)%[a]	(23.8 ± 4.1)%[b]	(4.2 ± 6.5)%[c]
6		*ri*	(16.7 ± 5.0)%[a]	(30.3 ± 6.9)%[b]	(5.4 ± 10.4)%[c]
7		*nr*	(26.2 ± 1.2)%[a]	(67.8 ± 2.0)%[b] K_i = 27.9 ± 3.9 μM[e]	(14.9 ± 2.0)%[c]
8		*nr*	(30.4 ± 9.6)%[a]	(56.5 ± 4.0)%[b] K_i = 33.8 ± 3.5 μM[d]	(10.2 ± 3.3)%[c]

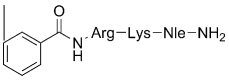
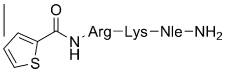
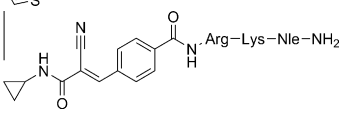
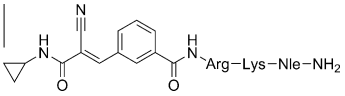
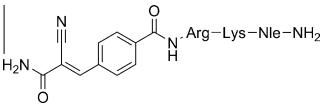
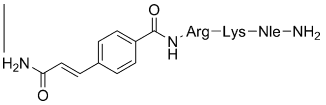
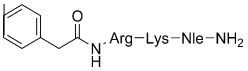
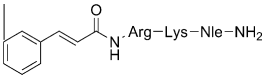
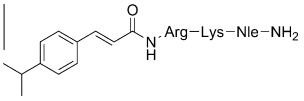
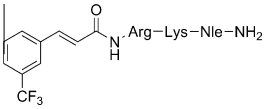
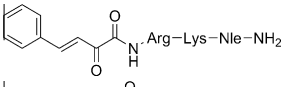
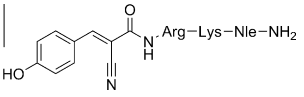
[a] % Inhibition of the Dengue Virus (DEN) NS2B-NS3 protease (enzyme: 100 nM; inhibitor 50 μM; substrate 50 μM).
[b] % Inhibition of the West Nile Virus (WNV) NS2B-NS3 protease (enzyme: 150 nM; inhibitor 50 μM; substrate 50 μM).
[c] % Inhibition of thrombin (enzyme: 10 nM; inhibitor 25 μM; substrate 50 μM).
[d] K_i-value calculated for competitive inhibition mechanism (best fitting results).
[e] K_i-value calculated for mixed-model inhibition mechanism (best fitting results).
[f] Peptide connection type: *r*, retro; *ri*, retro-inverse; *sri*, semi retro-inverse; *nr*, non-retro; *n*, no category.

creation of potent and selective DEN protease inhibitors, and are superior to natural sequences or retro-inverse peptides. The selectivity of the dipeptide compounds between the two viral serine proteases is low, but the off-target thrombin is practically unaffected by these analogs. The most "potent" thrombin inhibitor, compound **2**, has a K_i of more than 100 μM at thrombin. In general, the selectivity can – as shown below – be increased by insertion of an additional norleucine residue in the tripeptide analogs (Section 3.2.).

3.2. Retro tripeptides – N-terminal cap analysis

In addition to the dipeptides, we synthesized and characterized different tripeptides shown in Tables 2 and 3 to explore the influence of an additional norleucine residue and different *N*-terminal caps. The known peptide sequence Bz-Nle-Lys-Arg-Arg-NH_2 without any electrophilic "warhead" has a K_i-value of 127.5 μM at the DEN protease (Yin et al., 2006a). The retro sequence of this peptide with one arginine in P_1 missing, Bz-Arg-Lys-Nle-NH_2 (**9**) shows considerably higher activity with a K_i-value of 15.6 μM and a significant selectivity in comparison to human thrombin and WNV protease (Table 2). The benzoyl substituent apparently interacts with molecular recognition elements of the catalytic center of the DEN protease and may therefore be a replacement for the first arginine residue in P_1 position, which is an essential part in non-retro peptidic inhibitors. The fact that this tripeptide sequence is highly active and selective, while having a twisted backbone with an inverted *C*- and *N*-terminus, is extremely surprising. The twisted backbone is a consequence of the interchanged termini of the peptide and results in a contrary (flipped) orientation of the side chains (without an influence towards the stereocenters) and the carbonyl and amine parts of the amide bonds. The derivatisation of the benzoyl cap showed further interesting results. An alternative thiophene substituent in compound **10** leads to a small increase in activity (K_i = 10.6 μM at DEN protease). An expansion of the aromatic ring by a cyanoacrylamide system in compound **11** leads to a threefold increase of activity (K_i = 4.9 μM), accompanied by a similar selectivity profile with only marginal activity at the WNV protease (K_i = 92.5 μM) and thrombin. This result is in agreement with the dipeptides and underlines our previous reasoning that 3-aryl-2-cyanoacrylamides are preferred structures for an interaction with the active center of the protease, with the possibility of an interaction between the catalytic active serine and the nitrile acting as a weak electrophile (Nitsche et al., 2011). In contrast to the dipeptides we found no significant activity difference between *meta*- and *para*-substituted aromatic systems (**11** vs. **12**). Also in agreement with our previous results (Nitsche et al., 2011) we found no significant influence of the amide substituent (**11** vs. **13**), which offers the possibility to tune the lipophilicity of the peptide by choice of the amide substituent without any negative influences towards the activity. We observe only a minor difference in activity of the nitrile-containing derivative **13** and the "nitrile-free" analog **14**, indicating that the nitrile is not an essential part of these structures and that an electrophilic moiety is not mandatory to establish high affinities to the DEN protease.

Table 2
Tripeptides of the sequence R-Arg-Lys-Nle-NH_2 with different *N*-terminal cap groups.

No.	Structure	Type[f]	DEN	WNV	Thrombin
9		*r*	(68.1 ± 0.8)%[a] K_i = 15.6 ± 1.1 μM[d]	(7.7 ± 7.1)%[b]	(5.5 ± 7.1)%[c]
10		*r*	(71.1 ± 2.4)%[a] K_i = 10.6 ± 0.9 μM[d]	(14.7 ± 6.2)%[b]	n.i.
11		*r*	(88.5 ± 2.8)%[a] K_i = 4.9 ± 0.3 μM[d]	(28.0 ± 3.7)%[b] K_i = 92.5 ± 34.7 μM[e]	(13.4 ± 4.3)%[c]
12		*r*	(83.4 ± 2.2)%[a] K_i = 6.4 ± 0.4 μM[d]	(21.3 ± 2.4)%[b]	(7.6 ± 3.9)%[c]
13		*r*	(85.6 ± 2.9)%[a] K_i = 6.5 ± 0.6 μM[d]	(25.5 ± 8.8)%[b]	n.i.
14		*r*	(84.6 ± 2.5)%[a] K_i = 10.5 ± 0.8 μM[d]	(15.6 ± 3.2)%[b]	n.i.
15		*r*	(48.1 ± 3.3)%[a] K_i = 55.3 ± 7.0 μM[d]	n.i.	n.i.
16		*r*	(82.9 ± 6.3)%[a] K_i = 11.9 ± 0.9 μM[d]	(13.8 ± 7.3)%[b]	n.i.
17		*r*	(72.6 ± 1.2)%[a] K_i = 16.2 ± 0.7 μM[d]	(20.4 ± 1.1)%[b]	n.i.
18		*r*	(68.2 ± 0.7)%[a] K_i = 22.5 ± 1.3 μM[d]	(14.2 ± 2.8)%[b]	(8.7 ± 4.8)%[c]
19		*r*	(69.5 ± 8.8)%[a] K_i = 9.5 ± 1.6 μM[d]	(23.0 ± 2.5)%[b]	(10.8 ± 1.7)%[c]
20		*r*	(56.7 ± 0.9)%[a] K_i = 31.3 ± 10.2 μM[e]	(46.7 ± 2.0)%[b]	(16.3 ± 0.4)%[c]

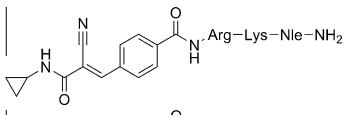
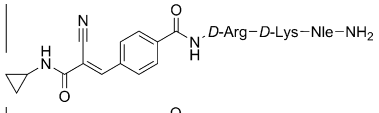
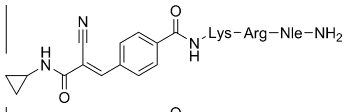
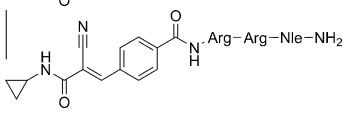
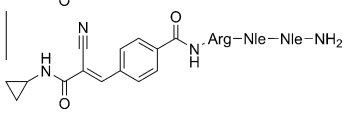
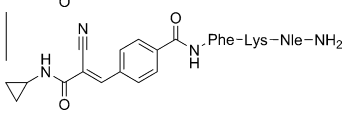
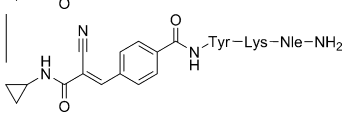
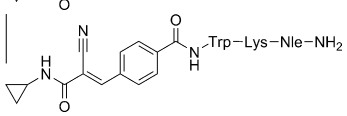
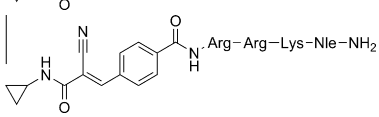
n.i., no inhibition.
[a] % Inhibition of the Dengue Virus (DEN) NS2B-NS3 protease (enzyme: 100 nM; inhibitor 50 μM; substrate 50 μM).
[b] % Inhibition of the West Nile Virus (WNV) NS2B-NS3 protease (enzyme: 150 nM; inhibitor 50 μM; substrate 50 μM).
[c] % Inhibition of thrombin (enzyme: 10 nM; inhibitor 25 μM; substrate 50 μM).
[d] K_i-value calculated for competitive inhibition mechanism (best fitting results).
[e] K_i-value calculated for mixed-model inhibition mechanism (best fitting results).
[f] Peptide connection type: *r*, retro; *ri*, retro-inverse; *sri*, semi retro-inverse; *nr*, non-retro; *n*, no category.

Nevertheless, the nitrile-containing structures are the most active and selective ones tested in this series and they are also easily accessible (in comparison to **14**) by a simple one-step Knoevenagel condensation followed by solid phase synthesis.

Schüller et al. (2011) found the phenylacetyl cap group as a part of the most active inhibitor for DEN protease in the studied tripeptidyl aldehyde inhibitors. In our series, the phenylacetyl cap (compound **15**) has the weakest affinity towards the DEN protease with a K_i-value of 55.3 μM. In analogy to our previous results, the cinnamyl double bond is an essential part of the pharmacophore: the cinnamyl derivatives **16**, **17** and **18** show much higher activities than the saturated compound **15**. In analogy to Steuer et al. (2011) the electrophilic ketoamide **19** was synthesized, which also shows good activity with a K_i-value of 9.5 μM against the DEN protease. Compound **20** contains an arylcyanoacrylamide that is reversely connected to the peptide (cf. Nitsche et al. (2011)). This derivative shows lower activity and selectivity between the different serine proteases tested (K_i = 31.3 μM at DEN protease). Considering these results, it is obvious that the connection of the peptide residue via the aromatic ring is preferred, as compared to the connection via the acrylic acid function.

An extension of the dipeptides (Table 1) with a norleucine residue (Table 2) leads to a significant change in the activity profile of the compounds: the affinity towards DEN protease increases significantly, whereas nearly all affinity towards WNV protease is lost. For example, the benzoyl capped tripeptide **9** (Table 2) shows a considerable increase of activity at the DEN protease (K_i = 15.6 μM vs. K_i = 39.6 μM), accompanied by a further decrease of activity at the WNV protease in comparison to the dipeptide **1** (Table 1). A comparison of the arylcyanoacrylamide tripeptide **11** and the dipeptide analog **2** underlines the change in the activity profile by addition of the norleucine residue. Against Dengue protease there

Table 3
Tri- and tetrapeptides – sequence derivatisation.

No.	Structure	Type[f]	DEN	WNV	Thrombin
11		*r*	(88.5 ± 2.8)%[a] K_i = 4.9 ± 0.3 μM[d]	(28.0 ± 3.7)%[b] K_i = 92.5 ± 34.7 μM[e]	(13.4 ± 4.3)%[c]
21		*sri*	(24.1 ± 1.1)%[a]	(22.3 ± 2.6)%[b]	(8.4 ± 4.3)%[c]
22		*n*	(24.7 ± 5.5)%[a]	(22.4 ± 2.6)%[b]	(6.9 ± 3.0)%[c]
23		*n*	(29.1 ± 2.2)%[a]	(22.5 ± 3.0)%[b]	(12.4 ± 4.2)%[c]
24		*n*	(26.4 ± 3.3)%[a]	(28.9 ± 6.7)%[b]	(4.8 ± 2.0)%[c]
25		*n*	(41.0 ± 4.7)%[a]	n.i.	n.i.
26		*n*	(25.3 ± 4.0)%[a]	(16.8 ± 2.8)%[b]	n.i.
27		*n*	(38.7 ± 3.4)%[a]	(16.1 ± 1.6)%[b]	n.i.
28		*r*	(75.0 ± 3.3)%[a] K_i = 15.0 ± 1.1 μM[d]	(42.3 ± 4.0)%[b] K_i = 42.0 ± 7.5 μM[e]	n.i.

n.i., no inhibition.
[a] % Inhibition of the Dengue virus (DEN) NS2B-NS3 protease (enzyme: 100 nM; inhibitor 50 μM; substrate 50 μM).
[b] % Inhibition of the West Nile Virus (WNV) NS2B-NS3 protease (enzyme: 150 nM; inhibitor 50 μM; substrate 50 μM).
[c] % Inhibition of thrombin (enzyme: 10 nM; inhibitor 25 μM; substrate 50 μM).
[d] K_i-value calculated for competitive inhibition mechanism (best fitting results).
[e] K_i-value calculated for mixed-model inhibition mechanism (best fitting results).
[f] Peptide connection type: *r*, retro; *ri*, retro-inverse; *sri*, semi retro-inverse; *nr*, non-retro; *n*, no category.

is an activity increase from a K_i-value of 29.0 to 4.9 μM, whereas against the WNV protease a decrease of activity from 26.4 μM to 92.5 μM occurs. Very important is the fact that nearly all explored tripeptides show no relevant activity towards human thrombin, which is a basic requirement for advanced therapeutic investigations.

Compound **11** is the most active compound in this series. It is possible that the arylcyanoacrylamide moiety acts as a mimetic of the first arginine in P_1 position near the active center of the protease, whereas the tripeptide sequence occupies other protein pockets, for example S_2–S_4. This supposed interaction requires the inversion of the peptide inhibitor's *C*- and *N*-termini. Thus, the backbone would be inverted and the L-amino acid side chains arranged in opposition to the side chains of the normal (non-retro) peptide as described before. Alternatively, the aromatic cap group could also be located near the S_3 or the S_1' pocket, with the arginine and lysine residues binding to the S_1 and S_2 pockets. However, these binding modes are difficult to combine with our further results (Section 3.3.): for example, analog **23**, containing two arginine residues (R-Arg-Arg-Nle), shows no relevant activity. To obtain additional information about the binding mechanism and to exclude a promiscuous binding mode, we used the fluorescence quenching assay described by Bodenreider et al. (2009) to show that the arylcyanoacrylamide group is acting near the active site of the DEN protease. This competition assay is based on the known inhibitor aprotinin and the intrinsic fluorescence of Trp_{50}, which is located near the active center of the protein. The fluorescence of this tryptophan is stimulated at a wavelength of 280 nm and quenched by substances that bind to the active site and have an UV absorption near 330 nm. Compound **11** shows an absorption maximum near 330 nm in the used assay buffer, and is therefore qualified for this experiment. Responsible for the absorption around 330 nm of **11** is only the arylcyanoacrylamide substituent. Thus, a significant decrease of the fluorescence with increasing inhibitor concentration argues for a localization of the aromatic moiety within a radius of 10–15 Å (Förster distance for FRET) from Trp_{50}. This residue is close to the substrate binding region. If the assayed inhibitor is displaced by aprotinin, the fluorescence is partially restored, as could also be shown for compound **11** (Fig. 2). This indicates that there is a competition between the two inhibitors at the active site of the protease, and therefore an interaction of the peptidic inhibitor with the active center of the DEN protease. However, there is no certainty regarding the

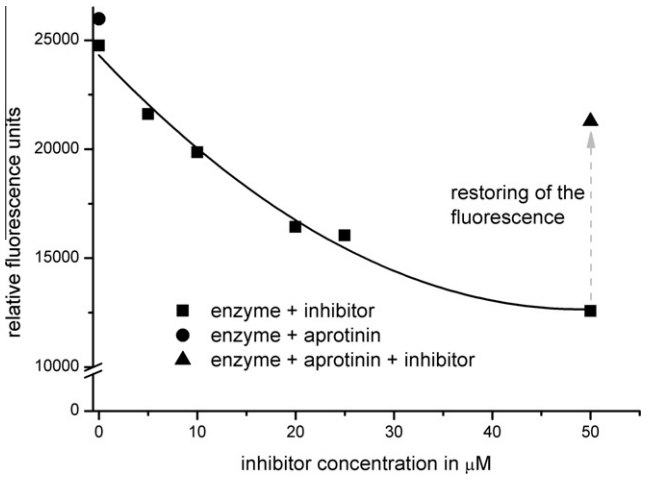


Fig. 2. Aprotinin competition assay of compound **11**. The variation limits of all measurements were below 5%.

location of the aryl component. For an efficient FRET, most substrate binding pockets are sufficiently close to Trp_{50}. Because of the structure–activity relationships presented here, we assume that the aromatic moiety binds to the S_1 or S_1' pocket, whereas the Arg-Lys-Nle tripeptide sequence occupies the S_2–S_4 pockets.

3.3. Retro tripeptides – sequence analysis

In addition to the *N*-terminal caps we explored different peptide sequences for the most active arylcyanoacrylamide cap (Table 3). In analogy to the results described before with the dipeptides, there is a loss of activity and selectivity when D-arginine and D-lysine are used in the semi retro-inverse peptide **21**. An interchange of the lysine and arginine in compound **22** and a replacement of the lysine by a second arginine residue in compound **23** leads to a pronounced decrease of activity at the DEN protease, along with an unchanged selectivity profile towards WNV protease and thrombin. This finding supports the hypothesis that the arginine and lysine residues bind at the S_2 and S_3 and not the S_1 and S_2 pockets. If there is an interaction of the arginine residue in S_1 and the lysine in S_2 we would expect an increase or at least a consistence of activity by replacing the lysine residue with an arginine (**11** vs. **23**), because two arginine residues or generally two basic amino acids are preferred in the P_1 and P_2 positions. This could recently be confirmed by the first crystal structure of a tetrapeptide–aldehyde inhibitor with the DEN serotype 3 protease (Noble et al., 2012), where the two arginine residues bind at the S_1 and S_2 pockets. The inferior activity of cpd. **22** in comparison to cpd. **11** again underlines the preference for arginine in P_2 and lysine in P_3 at the DEN protease. A replacement of the lysine by norleucine (**24**) also leads to a nearly inactive molecule. Additionally we evaluated the tetrapeptide **28** with two arginine residues (R-Arg-Arg-Lys-Nle), which shows only one third of the activity and lower selectivity than **11** containing only one arginine residue, indicating once more that there is no absolute requirement for two arginine residues in peptidic DEN protease inhibitors.

Highly basic and polar functionalities such as the guanidine group in arginine residues are often associated with poor bioavailability. Because of this, our approach aims to replace at least the arginine residue in P_1 with a more drug-like moiety (cap group). Yin et al. (2006b) also investigated alternative amino acid residues for the P_1 position, by exploring tryptophan, phenylalanine and derivatives with activity results similar to the arginine (P_1) substituted tetrapeptidyl aldehydes (Bz-Nle-Lys-Arg-Arg-H). For the development of pharmaceutically interesting molecules the second arginine residue in P_2 should also be replaced by a more drug-like group. As a first analysis of this problem we explored alternative amino acids for the arginine residue at the most potent inhibitor in this series (**11**), which are shown in Table 3. The replacement of the arginine by phenylalanine (**25**) or tryptophan (**27**) is more promising than by tyrosine (**26**), but nevertheless leads to a loss of activity, which indicates that in future work more complex side chains must be explored to replace this arginine residue.

4. Conclusion

In this work we present the first potent peptide-based inhibitors of the DEN protease that do not require a highly electrophilic "warhead". These inhibitors are retro-peptides with inverted *N*- and *C*-termini. The most active molecules are retro-tripeptides based on the sequence R-Arg-Lys-Nle-NH_2 with an arylcyanoacrylamide group as *N*-terminal cap. The norleucine residue is essential for the activity and selectivity of the compounds. Therefore, for the first time DEN selective tripeptide inhibitors could be explored, which show only a negligible activity against other serine proteases. By the tryptophan fluorescence quenching assay we could exclude a promiscuous binding mode and demonstrate that the inhibitor binds near the active center of the enzyme. Considering the structure–activity relationships of the explored di-, tri- and tetrapeptides it is possible that the most active retro tripeptides (R-Arg-Lys-Nle-NH_2) are arranged with an inverted *C*- and *N*-terminus in the enzyme pockets S_1–S_4, with the aromatic *N*-terminal cap being located near the S_1 recognition residues of the protein, followed by the other side chains in the S_2–S_4 pockets. Future investigations will aim at the development of alternative structures for the arginine residue to further increase the drug-likeness. For this, numerous arginine mimetics are available (Peterlin-Mašič and Kikelj, 2001). We are confident that the affinity of the compounds presented here can be increased in future and ongoing work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2012.02.008.

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