

Strategies towards protease inhibitors for emerging flaviviruses

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Abstract

Infections with flaviviruses are a continuing (dengue) or recently emerged (Zika) global threat. In addition to vaccine development and vector controls, antiviral agents that alleviate symptoms in patients are of considerable interest. Among others, the protease NS2B-NS3 is a promising drug target to inhibit viral replication. Flaviviral proteases share a high degree of similarity regarding structure and substrate-recognition, which may facilitate a strategy towards pan-flaviviral protease inhibitors. However, the success of various drug discovery attempts during the last decade has been limited by the nature of the viral enzyme as well as a lack of structural templates. Small-molecular, structurally diverse protease inhibitors reach affinities in the lower micromolar range. Peptide-based, substrate-derived compounds are often nanomolar inhibitors, however, with highly compromised drug-likeness. With some exceptions, the antiviral cellular activity of most of the reported compounds has been insufficient. Recent progress has been made in the elucidation of inhibitor binding using different structural methods. This will hopefully lead to more rational attempts for the identification of various lead compounds that may be successful in cellular assays, animal models and beyond.

Keywords

Protease, inhibitor, peptides, small-molecular, flavivirus, dengue, West Nile, Zika

1. The need for antivirals against flaviviruses

The inhibition of viral enzymes plays an outstanding role in antiviral therapy. In particular, if efficient vaccines are not available and vector control struggles, agents that reduce symptoms in patients by inhibiting the viral replication process are the main option. Recent progress in flaviviral vaccine development currently faces crucial challenges with non-predictable outcome. The serotype-related cross reactivity with human antibodies has been one of the main difficulties. Dengue virus exists in four serotypes, which have widely spread over all endemic areas within the last decades. An additional infection after a previous one with a different serotype can cause severe life-threatening symptoms, such as dengue haemorrhagic fever or shock syndrome. This caveat requires vaccines that simultaneously create pronounced immunity against all four serotypes. Since late 2015, the first vaccine CYD (Dengvaxia), which efficiency significantly varies among dengue serotypes, has been approved in several countries. However, recent studies have demonstrated that the effect of cross reactivity is not limited to the four known dengue serotypes. Immunity to dengue can cause antibody-dependent enhancement of Zika virus infections and potentially increases viremia and severity of the disease (Dejnirattisai et al. 2016; Priyamvada et al. 2016; Castanha et al. 2017). Consequently, it cannot be ruled out that treatment with dengue vaccine may increase the chance of a later enhanced Zika virus infection. Due to such unexpected phenomena, the extensive search for effective antiviral drugs should be promoted in addition to vaccine development. One important approach aims on inhibiting viral protease activity, as successfully demonstrated for chronic diseases such as hepatitis C or AIDS, where among others, inhibitors of HCV and HIV proteases are established in modern combination therapy.

2. Flaviviral proteases as drug targets

Due to limited druggability, all campaigns towards clinically relevant protease inhibitors for emerging flaviviruses have so far been unsuccessful. However, as throughout the flaviviral protease family a high degree of similarity in shape, substrate recognition and catalytic function is conserved, a successful drug development process may lead to pan-flaviviral protease inhibitors, which could be used against several globally challenging infectious diseases, such as dengue, West Nile or Zika fever. Therefore, the present chapter

aims on briefly summarizing the progress that has been made and tries to derive strategies for future attempts. Within in the last decade, a particular focus has been on the proteases of dengue and West Nile viruses. The highlights for these two most prominent examples will be discussed in this chapter. For a more comprehensive and detailed analysis regarding anti-invectives for these two viruses the interested reader is kindly referred to recent reviews (Behnam et al. 2016; Lim and Shi 2013; Lim et al. 2013; Nitsche et al. 2014). The chapter will also highlight recent campaigns for the identification of suitable inhibitors for Zika virus protease. Consequences from all these attempts will outline general perspectives for a more efficient and hopefully successful search for drug-like protease inhibitors for flaviviruses.

3. Function and structure of flaviviral proteases

Flaviviruses consist of a single-stranded positive-sense RNA genome, which is translated into a single polyprotein by the host cell's ribosomal system. The polyprotein comprises three structural (C, prM, E) and eight non-structural (NS) proteins, which have to be released from the polyprotein after selective protease cleavage (Lescar et al. 2008; Nitsche et al. 2014; Chappell et al. 2008). This essential posttranslational procession is executed by host and flaviviral proteases at the membrane of the endoplasmatic reticulum. The viral protease complex comprises a protease unit, located at the N-terminal part of NS3 and requires a hydrophilic core fragment of the membrane-associated NS2B as cofactor for catalytic activity. Highly conserved residues S135, His51 and Asp75 assemble the catalytic triad. The flaviviral serine NS2B-NS3 endoproteases show a common tendency to cleave peptidic backbones after two basic residues. However, detailed substrate preferences as well as catalytic efficiency vary among different flaviviruses.

Several crystal structures of dengue and West Nile proteases have been reported during the last decade. The main deviations are related to the role of the cofactor NS2B. Some structures reported NS2B to be disordered (referred as open or inactive form) whereas others resolved the cofactor domain wrapped around the active site of NS3 (referred as closed or active form). NMR studies indicate that, regardless of the presence or absence of ligands, the closed conformation is predominant (Su et al. 2009; de la Cruz et al. 2014). The importance of NS2B towards correct folding of the disordered NS3 domain has also been demonstrated (Gupta et al. 2015). Unfortunately only a limited number of co-crystal

structures with ligands or inhibitors as reliable basis for rational drug design are available. None of those comprise a small-molecular drug-like inhibitor. The first X-ray crystal structures of dengue and West Nile virus proteases became available in 2006 (Erbel et al. 2006). In case of West Nile virus this structure showed the catalytically active closed conformation with a substrate-derived tetrapeptidic aldehyde inhibitor covalently bound to Ser135 (pdb code: 2FP7). In case of dengue serotype 2 only an inactive open protease form without ligand could be crystallized (pdb code: 2FOM). Although inadequate, the latter structure was used as basis for several drug discovery campaigns until a closed and active form of dengue protease serotype 3 with the same tetrapeptidic aldehyde inhibitor became available in 2012 (pdb code: 3U1I) (Noble et al. 2012).

Recently, the first crystal structure of Zika virus protease in the active form with a boronate inhibitor, which suits a reasonable model for rational drug discovery campaigns, could be solved (pdb code: 5LC0) (Lei et al. 2016). A second crystal structure without inhibitor revealed the open form (apo) of the enzyme with missing resolution for the C-terminal part of NS2B (pdb code: 5GXJ) (Chen et al. 2016b). A significantly divergent conformation was also obtained for an NS3 loop region between residues 152 and 167, which contributes to the S_1 shape. The obvious deviations between these initial crystal structures suggested a conformational activation upon substrate or inhibitor binding. Additional crystallographic and in-solution experiments were necessary to analyse these results in more detail. Since, several new crystal structures and NMR studies in presence and absence of ligands, covering ‘pre-open’ (pdb code: 5T1V), open and closed conformations have been reported (Lee et al. 2017; Phoo et al. 2016; Zhang et al. 2016; Mahawaththa et al. 2017). They discovered, in contrast to previous studies with dengue and West Nile protease, a more delicate dependence of open and closed conformations from the construct that was used to fuse NS2B and NS3. The artificial covalently linked construct gZiPro only adopts the closed conformation in presence of substrate-like ligands, but can also bind inhibitors in the open conformation (Mahawaththa et al. 2017). A construct with an autocleavage site between NS2B and NS3 (eZiPro) showed the closed conformation in the crystal structure (pdb code: 5GJ4), but NMR relaxation data indicated high mobility of NS2B in solution (Phoo et al. 2016). Apparently, a C-terminal tetrapeptide of NS2B that was found to occupy the active site could not maintain the closed state in solution. A construct without covalent linkage (bZiPro) displayed the closed conformation in solution and in the single crystal (pdb code: 5GPI) (Zhang et al. 2016). This construct could even be used to capture the structure of a

fragment hit in the closed state after being soaked into the crystal (pdb code: 5H4I) (Zhang et al. 2016). Consequently, bZiPro represents the most suitable construct for compound screening in addition to gZiPro, which may be superior to identify compounds that are able to bind to the open conformation or supposedly perturb the interaction between NS2B and NS3.

4. Inhibitors of flaviviral proteases

In case of dengue protease, which by far has been the most prominent and extensively studied example, several screening campaigns and related inhibitor development approaches were not able to identify promising lead compounds during the last decade (Nitsche et al. 2014). The main reasons for failure have been a lack of structural basis, a relatively flat binding site and a particular focus on often not rationally designed small-molecular compounds. Although viral proteases recognize peptidic substrates, only the minority of studies dealt with peptide-based inhibitors, which have shown to be the only class of compounds that can reach sufficient inhibition in the nanomolar concentration range. However, in contrast to the HIV and HCV success stories, the preference for two permanently charged basic side chains in flaviviral substrates complicates the development of drug-like peptide-derived inhibitors with sufficient bioavailability and antiviral activity in cell culture and animal models.

Table 1: Biochemical and cellular activities of selected flaviviral protease inhibitors discussed in this chapter.

Compound	Dengue virus [μM] ^a		West Nile virus [μM]		Zika virus [μM]	
	Biochemical ^b	Cellular	Biochemical ^b	Cellular	Biochemical ^b	Cellular
1	IC ₅₀ = 1.1	inactive				
2	K _i = 2.0		K _i = 4.6			
3	IC ₅₀ = 2.0	EC ₅₀ = 59.5 CC ₅₀ = 135	IC ₅₀ = 8.7	EC ₅₀ = 42.4 CC ₅₀ = 135		
4	IC ₅₀ = 2.2					
5	IC ₅₀ = 1.0	EC ₅₀ = 0.8 CC ₅₀ > 10				
6	IC ₅₀ = 0.5					
7	IC ₅₀ = 15.4	EC ₅₀ = 0.17 CC ₅₀ = 29.3				
8	IC ₅₀ = 1.2	EC ₅₀ = 39.4 CC ₅₀ > 100				
9	IC ₅₀ = 8.5		IC ₅₀ = 0.11			
10	IC ₅₀ = 2.8	EC ₅₀ = 40 CC ₅₀ = 213	IC ₅₀ = 0.26	EC ₅₀ = 42.3 CC ₅₀ = 213	IC ₅₀ = 1.1	
11	IC ₅₀ > 10	EC ₅₀ = 81.5 CC ₅₀ = 236	IC ₅₀ = 0.44	EC ₅₀ = 17 CC ₅₀ = 236	IC ₅₀ > 10	
12			IC ₅₀ = 1.1			
13					K _i = 9.5	
14	inactive	EC ₅₀ = 0.8 CC ₅₀ = 54			IC ₅₀ = 21.6	EC ₅₀ = 13.0 CC ₅₀ > 40
15	IC ₅₀ > 10	EC ₅₀ > 100 CC ₅₀ = 257	IC ₅₀ = 0.74	EC ₅₀ = 107 CC ₅₀ = 257	IC ₅₀ = 0.82	EC ₅₀ ~ 50 CC ₅₀ = 257
16	K _i = 0.051	EC ₅₀ = 30 CC ₅₀ > 100	K _i = 0.082	EC ₅₀ = 38 CC ₅₀ > 100	K _i = 0.040	
17	K _i = 0.078	EC ₅₀ = 19 CC ₅₀ > 100	K _i = 0.16	EC ₅₀ > 50 CC ₅₀ > 100	IC ₅₀ = 2.1	
18	K _i = 0.012	EC ₅₀ = 20 CC ₅₀ > 100	K _i = 0.039	EC ₅₀ = 23 CC ₅₀ > 100		
19	IC ₅₀ = 0.028	EC ₅₀ = 7.1 CC ₅₀ > 100	IC ₅₀ = 0.12		IC ₅₀ = 1.0	
20	IC ₅₀ = 0.18	EC ₅₀ = 3.4 CC ₅₀ = 100	IC ₅₀ = 0.56	EC ₅₀ = 15.6 CC ₅₀ > 100		

^a Activities have been reported for various serotypes. The serotype with the best activity results is reported here.

^b If reported activities vary by method or report, the lowest (best) value is shown.

4.1. Small-molecular dengue virus protease inhibitors

Approximately 40 approaches towards small-molecular non-peptide-derived inhibitors have been reported in the literature during the last decade. Unfortunately, only a minority of them did provide cellular data to confirm that the compounds also achieve proper antiviral activity in cells and are not cytotoxic. A remarkable number of compounds with broad structural variety that are able to inhibit dengue (and West Nile) protease in the concentration range between 25 and 100 μM have been identified. Further ligand derivatizations often led to limited improvements with maximal affinities in the one-digit micromolar range. Examples are rare where a small structural change causes a pronounced impact on affinity, a phenomenon, which is well known from other drug development campaigns as activity cliff. Consequently, no studies could provide small-molecular non-peptidic compounds that are able to bind to dengue protease in the desirable lower nanomolar range.

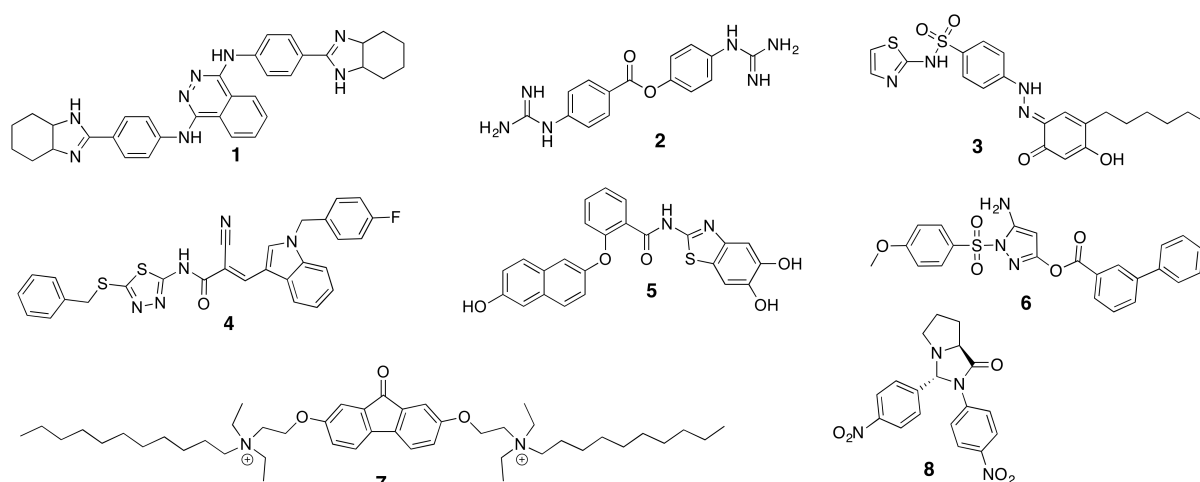


Fig. 1: Small-molecular inhibitors of dengue virus protease, which either reached highest affinity in biochemical or phenotypic assays.

Compound **1** resulted from a campaign including high-throughput screening, scaffold optimization and subsequent derivatization (Bodenreider et al. 2009). This compound has an IC_{50} of 1.1 μM against dengue serotype 2 and decreased potency towards the other serotypes. Specific competitive binding was confirmed by several orthogonal methods; however, despite strong efforts no further optimization of this compound class, which also lacked antiviral activity in cell culture, could be achieved (Lim et al. 2013). Compound **2** was identified from a computational screening approach (Knehans et al. 2011) with a K_i value of 2.0 μM

(serotype 2). Both guanidine groups are crucial for sufficient activity, indicating a likely electrostatic interaction with residues in the S₁ and S₂ pockets of the protease. A covalent interaction of the activated ester bond with Ser135 is possibly, but has not yet been confirmed experimentally. Compound **3** is supposedly an allosteric inhibitor, identified from a virtual screening of compounds, actually aiming on West Nile virus protease, which may inhibit the interactions between NS3 and NS2B (Shiryaev et al. 2011). It shows an IC₅₀ value of 2.0 μM, some basic antiviral activity in cells and limited cytotoxicity. A structurally similar but larger derivative **10** (Fig. 2) identified from the same campaign showed only slightly lower affinity (IC₅₀ = 2.8 μM), but improved cellular data (EC₅₀ = 40 μM). From a series of thiadiazoloacrylamides, compound **4** showed best activity with an IC₅₀ of 2.2 μM (K_d = 2.1 μM) (Liu et al. 2014). In correlation with various previously studied compound series the SAR between derivatives remained remarkably flat.

For compound **5** IC₅₀ values of up to 1.0 μM (serotype 3) from a biochemical and 3.2 μM from a cell-based protease assay have been reported (Wu et al. 2015). The compound shows cellular antiviral activity in the same range (EC₅₀ = 0.8 μM) but also cytotoxic effects at concentrations above 10 μM. The most active small-molecular non-peptidic inhibitor reported so far for dengue protease is compound **6**. It is one of the few examples with affinity significantly lower than 1 μM with an IC₅₀ value of 0.5 μM for serotype 2 (Koh-Stenta et al. 2015). It is also one of the few examples that aim at targeting the catalytically active serine by a covalent interaction. This could be proven by mass spectrometry and may be the key towards highly affine small ligands. After reaction, the biphenyl-3-carboxylate remains bound to the protease and blocks all further substrate procession until ester hydrolysis may restore the protease activity again.

Another high-throughput screen identified amphiphilic compound **7** with only moderate activity in the biochemical assay (IC₅₀ = 15.4 μM) (Yang et al. 2011). However, cellular assays revealed one of the highest reported antiviral activities in cells for any discovered small-molecular dengue protease inhibitor (EC₅₀ = 0.17 μM). Resistance breeding experiments suggested an inhibition of interactions between the NS2B and NS3 domains. The remarkable discrepancy between biochemical and cellular activity may, however, indicate that the protease is not the only target of this compound. An independent screening campaign based on a dengue replicon assay identified a structurally similar compound with related biochemical and virological results (Yang et al. 2014).

Recently, further derivatizations (including rigidification) of previously published methionine-proline anilides (Zhou et al. 2013) towards non-peptidic analogues revealed compound **8** as small-molecular dengue protease inhibitor with pronounced affinity ($IC_{50} = 1.2 \mu\text{M}$) (Weng et al. 2016). Selective interaction with the protease is supported by the inactivity of a stereoisomer of **8**. In combination, both SARs indicate that the two aromatic nitro substituents are necessary for proper affinity. Although this functional group is highly questionable in terms of drug-likeness, the compound showed no cytotoxic effects at the highest assayed concentration of $100 \mu\text{M}$ and was proven to inhibit viral replication in cell culture ($EC_{50} = 39.4 \mu\text{M}$).

Tolcapone, tannic acid and suramin have been reported as hits from a high-throughput screening with K_i values significantly below $1 \mu\text{M}$ (Balasubramanian et al. 2016). Tannic acid ($K_i = 0.34 \mu\text{M}$) showed also exceptional activity in a viral plaque assay with an EC_{50} value of $0.084 \mu\text{M}$ and only limited cytotoxic effects. However, with a molecular mass of 1700 Da and a polyphenolic structural characteristic this compound would usually not be considered as a suitable lead in drug discovery.

4.2. Small-molecular West Nile virus protease inhibitors

Most of the remarks and conclusions that have been made for the development of dengue virus protease inhibitors can be transferred to the closely related West Nile virus protease. In fact, only a limited number of studies directly aimed for identifying West Nile virus protease inhibitors. However, often West Nile protease activity was additionally assessed within dengue protease inhibitor campaigns. Compound **2** (Fig. 1) for example was found to be notably active against West Nile virus protease ($K_i = 4.6 \mu\text{M}$), although the computational screening approach based on a homology model of dengue protease. The highest affinities that could be reached with small-molecular compounds were often up to one order of magnitude better compared to dengue protease.

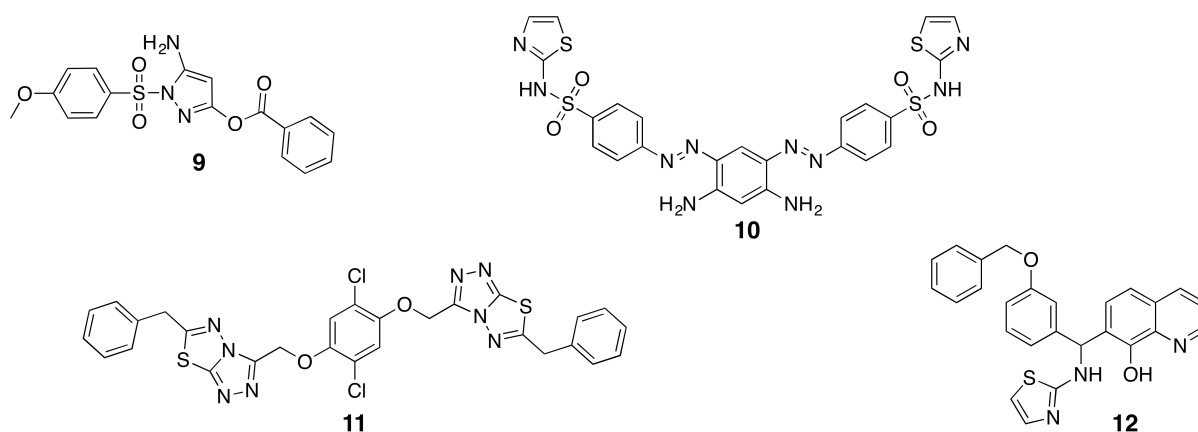


Fig. 2: Selected examples of small-molecular compounds that reached highest inhibition activities against West Nile virus protease.

Analogues of compound **6** (Fig. 1) have been studied for West Nile virus protease before they were evaluated for dengue protease (Johnston et al. 2007; Sidique et al. 2009). For derivate **9** promising IC_{50} values between 0.11 and 0.16 μM have been reported (only 8.5 μM for dengue protease) (Koh-Stenta et al. 2015; Johnston et al. 2007). These covalently binding pyrazole esters can be considered as the class of small-molecular compounds with the highest reported affinities and ligand efficiencies for dengue and West Nile virus proteases. However, due to general high reactivity, their chemical stability even in usual assay buffer is limited (Sidique et al. 2009). Compounds **10** and **11** were identified from the same virtual screening campaign as compound **3** with IC_{50} values of 0.26 and 0.44 μM as well as moderate antiviral activity in cells with EC_{50} values of 42 and 17 μM , respectively (Shiryayev et al. 2011). From a high-throughput screening a compound comprising an 8-hydroxyquinoline scaffold was obtained as West Nile virus protease inhibitor with promising activity in cell culture ($EC_{50} = 1.4 \mu\text{M}$) (Mueller et al. 2008). Further derivatizations of this compound class resulted in **12** with an IC_{50} value of 1.1 μM (Ezgimen et al. 2012).

4.3. Small-molecular Zika virus protease inhibitors

Inspired by previous campaigns, initial progress has been made in the discovery of the first small-molecular inhibitors of Zika virus protease. Several compounds that resulted from dengue or West Nile virus protease screenings, such as **10** and **11**, also showed inhibition against Zika virus protease (Shiryayev et al. 2017). Compound **13** ($K_i = 9.5 \mu\text{M}$) is an example

of a series of lead compounds that have been recycled from an HCV protease high-throughput screening campaign (Lee et al. 2017). The dopamine antagonist bromocriptine (**14**), which has previously been reported to inhibit viral replication for all four dengue serotypes (Kato et al. 2016), also reduces Zika virus replication in cell culture (Chan et al. 2017). In contrast to dengue, the Zika virus protease could be confirmed as potential target of bromocriptine ($IC_{50} = 21.6 \mu M$). Compound **15**, which was previously reported to be active against West Nile ($IC_{50} = 0.74 \mu M$) but inactive against dengue protease (at concentrations lower than $10 \mu M$), was found to be also a promising Zika protease inhibitor ($IC_{50} = 0.82 \mu M$), especially from the perspective of ligand efficiency (molecular weight = 190 Da). It inhibited viral replication in cell culture ($EC_{50} \sim 50 \mu M$) and could reduce the level of circulating Zika viruses in mice (Shiryayev et al. 2017). Although these data are promising, apart from docking studies, which suggest binding close to the active site and interference with NS2B, structural data that would facilitate a hit-to-lead campaign are missing.

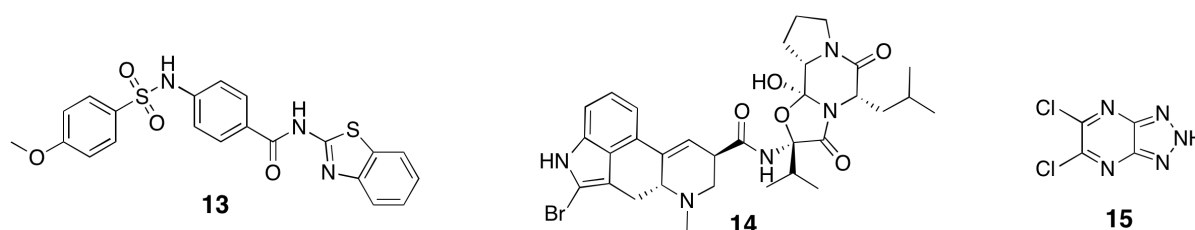


Fig. 3: Small-molecular inhibitors of Zika virus protease that resulted from previous campaigns for related viruses.

4.4. Peptide-derived inhibitors of dengue, West Nile and Zika virus proteases

Substrate-based peptidic inhibitors of dengue and West Nile virus proteases have been studied quite extensively during the earlier attempts. They usually consist of a substrate segment comprising at least two basic side chain residues and may additionally be featured with a C-terminal electrophilic warhead, most often an aldehyde moiety, for covalent binding to Ser135. They often cannot be considered as drug-like leads due to their unsuitable pharmacokinetic and physicochemical properties, such as bioavailability, specificity and plasma stability. Notably, these peptides could reach higher affinity towards West Nile than dengue virus protease. For oligo-D-arginines affinities up to 1 nM have been reported in case of West Nile virus (Shiryayev et al. 2006). Peptidomimetics with N-terminal dichloro-

substituted phenylacetyl groups and C-terminal arginine mimetics reached IC_{50} values of up to $0.12 \mu\text{M}$ (Hammamy et al. 2013). Peptides with an additional possibility for covalent interaction with Ser135 were able to generate affinities of up to 9 nM (K_i) for the tripeptidic aldehyde phenacetyl-Lys-Lys-Arg-H (Schüller et al. 2011; Stoermer et al. 2008; Knox et al. 2006). Although this compound showed serum stability, cell permeability and antiviral activity ($EC_{50} = 1.6 \mu\text{M}$) no further studies towards more drug-like derivatives have been reported (Stoermer et al. 2008).

In case of dengue virus protease, studies with simple substrate-derived peptides reached K_i values of only up to $0.3 \mu\text{M}$ (Prusis et al. 2013). Substrate-like peptides containing C-terminal aldehydes were less active in case of dengue protease with a K_i of $1.5 \mu\text{M}$ for the most active derivative Bz-Lys-Arg-Arg-H (Yin et al. 2006a). However, alternatively studied electrophiles such as the trifluoromethylketone in Bz-Nle-Lys-Arg-Arg- CF_3 ($K_i = 0.85 \mu\text{M}$) or a boronic acid function in a similar analogue Bz-Nle-Lys-Arg-Arg- $\text{B}(\text{OH})_2$ ($K_i = 0.043 \mu\text{M}$) showed increased activity (Yin et al. 2006b).

Recent studies have shown that even very small peptide-derived compounds can establish extraordinary binding affinities, if they are combined with such a boronic acid moiety, which forms a boronate with the catalytically active residue S135 (Nitsche et al. 2017). These compounds may not only offer a route towards more drug-like small-molecular derivatives, they have also become valuable tools in structural biology to elucidate inhibitor-protease interactions, which will hopefully illuminate the way towards a more structure-based drug design. Although, the drug-likeness of this compound class is limited, a significant reduction of viral titers for West Nile and dengue viruses in cell culture could be observed. Compound **16** showed pronounced pan-flaviviral protease affinity with K_i values of 51 , 82 and 40 nM for dengue, West Nile and Zika virus proteases, respectively. This derivative was co-crystallized with the proteases of Zika (Lei et al. 2016) and West Nile (Nitsche et al. 2017) viruses. Derivative **17** was used to demonstrate a novel NMR-based approach to identify the binding mode of tightly binding inhibitor molecules towards dengue virus protease from serotype 2 (Chen et al. 2016a). The *tert*-butyl moiety in **17** appears as a sharp and isolated signal in proton NMR spectra. Using the possibilities of paramagnetic NMR spectroscopy (Nitsche and Otting 2017) combined with NOEs, the positions of the *tert*-butyl group and aromatic protons in close proximity could be predicted in relation to the 3D structure of the

protein. Very recently, compounds **16** and **17** have been used to study the conformational flexibility of the NS2B cofactor of Zika protease in solution (Mahawaththa et al. 2017).

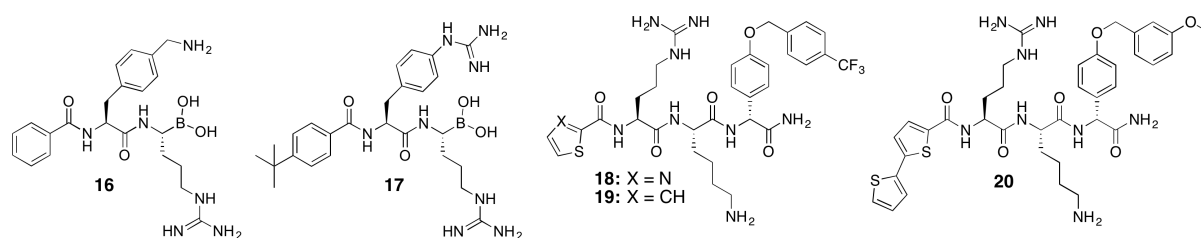


Fig. 4: Recently published highly affine peptide-derived inhibitors of flaviviral proteases.

Finally, over the last five years the stepwise elaboration of non-covalently binding tripeptidic inhibitors, comprising two basic side chains, has been reported regularly (Bastos Lima et al. 2015; Behnam et al. 2015; Behnam et al. 2014; Nitsche et al. 2012; Nitsche et al. 2013; Weigel et al. 2015). The optimization did so far only focused on enzymatic inhibition in biochemical assays, limiting the drug-likeness and pharmacokinetic properties of this compound class. However, with a K_i value of 12 nM, **18** is the compound of highest affinity towards dengue serotype 2 protease reported so far (Behnam et al. 2015). It also shows remarkable affinity against West Nile virus protease with a K_i of 39 nM. Due to limited permeability, the antiviral cellular activity is only moderate with EC_{50} values of 20 and 23 μ M for dengue and West Nile viruses, respectively. Recently, the highly related derivative **19** was analysed against Zika protease (Kuiper et al. 2017). It showed reduced inhibition potential for Zika ($IC_{50} = 1.0 \mu$ M) compared to previous reported results for dengue ($IC_{50} = 0.028 \mu$ M) and West Nile proteases ($IC_{50} = 0.12 \mu$ M) (Behnam et al. 2015; Kuiper et al. 2017). Increased cellular activity was found for analogue **20** with slightly improved permeability (dengue: $EC_{50} = 3.4 \mu$ M; West Nile: $EC_{50} = 15.6 \mu$ M) (Behnam et al. 2015). However, the affinity of **20** towards the proteases dropped significantly compared to **18** or **19** with IC_{50} values of 176 and 557 nM for dengue and West Nile proteases, respectively. Structural evidences, such as NMR or crystallographic data, for the binding mode of this compound class are unfortunately missing. This information would be very valuable to rationally understand the pronounced SAR.

4.5. Perspectives towards Zika virus protease inhibitors

All compounds that have recently been described to inhibit Zika protease, either small molecules or peptides, originated from previous drug discovery campaigns for related viruses. The co-crystal structures of compound **16** (Lei et al. 2016) and the fragment benzimidazol-1-ylmethanol (Zhang et al. 2016) in the active form present a unique chance for rationality in the upcoming drug discovery attempts. This opportunity was not available during a long period of early dengue protease inhibitor investigations. The main lessons for Zika learned from a decade of challenging dengue protease research are: to balance the focus between small-molecular and substrate-derived inhibitors; to take advantage of the virtue of covalence in enzyme inhibition; and to generate as much structural information as possible.

The latter aspect becomes obvious by taking a closer look into the co-crystal structure of Zika virus protease with compound **16**. In contrast to other flaviviral proteases, such as those from dengue or West Nile viruses, Zika comprises a negatively charged aspartate residue in position 83 of the cofactor domain NS2B. The West Nile protease contains a structurally very similar, although uncharged, asparagine residue in that position. Dengue protease serotypes have either a serine or threonine in that position. Without any structural information this situation may not have led to any special attention for drug development purposes with Zika virus protease. However, from the crystal structure it turned out that this NS2B aspartate residue is responsible for a salt-bridge formation with the aminomethyl-phenyl moiety (P₂) of compound **16** leading to an extra tight binding of inhibitors and substrates. As the possibilities for tight interactions of inhibitors with flaviviral proteases are usually limited, this information is highly relevant for further drug design campaigns.

5. Conclusion

Although during the last years several alternative flaviviral proteins (e.g. NS1 and NS5) as well as virus-host-interactions became attractive drug targets, the NS2B-NS3 protease is still of considerable interest. However, the progress towards drug-like compounds with promising intracellular activity was limited. It may take another decade until the first selective flaviviral protease inhibitor that convincingly works in an animal model will become available. The main challenges are the rather flat binding sites, the absence of product inhibition (in contrast to HCV) and the strong recognition preference for basic

moieties, which highly complicates the development of compounds with high affinity and a desirable ADME profile.

As new methods and technologies have also become available during the last decade of struggling inhibitor development, they may open the way for alternative strategies. Many of the earlier attempts especially towards small-molecular compounds were pursued without any reasonable structural basis. Co-crystal structures of flaviviral proteases with small-molecular ligands are still remarkably rare compared to other drug discovery campaigns. Technologies that do not rely on protease crystals, such as modern NMR, can help to elucidate the binding mode of inhibitor candidates. In this context, recently rising fragment-based screening approaches in combination with NMR, which have so far been neglected for flaviviral proteases, may also reanimate the drug discovery process. The identification of new small scaffolds with only weak affinity and their consequent rational elaboration into drug-like inhibitors may be superior compared to previous approaches.

In addition to new strategies regarding the identification and optimization of lead compounds, new approaches regarding cellular assays will be required to accelerate the present delay or total lack of cellular data. Some initial progress on intracellular protease assays has been made, but further development in this area, also concerning imaging techniques to track potential drug candidates within the cell, is necessary.

Note

Since the ‘Tofo Advanced Study Week on Arboviruses’ significant progress has been achieved particularly in the field of Zika virus protease. These recent results are reflected in this chapter, although they were not content of the conference presentation and discussion.

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