Accepted Manuscript

Pan-NS3 protease inhibitors of hepatitis C virus based on an R^3-elongated pyrazinone scaffold

Anna Karin Belfrage, Eldar Abdurakhmanov, Eva Åkerblom, Peter Brandt, Hiba Alogheli, Johan Neyts, U. Helena Danielson, Anja Sandström

PII: S0223-5234(18)30162-4

DOI: 10.1016/j.ejmech.2018.02.032

Reference: EJMECH 10209

To appear in: *European Journal of Medicinal Chemistry*

Received Date: 2 July 2017

Revised Date: 20 January 2018

Accepted Date: 10 February 2018

Please cite this article as: A.K. Belfrage, E. Abdurakhmanov, E. Åkerblom, P. Brandt, H. Alogheli, J. Neyts, U.H. Danielson, A. Sandström, Pan-NS3 protease inhibitors of hepatitis C virus based on an R^3-elongated pyrazinone scaffold, *European Journal of Medicinal Chemistry* (2018), doi: 10.1016/j.ejmech.2018.02.032.

Graphical Abstract

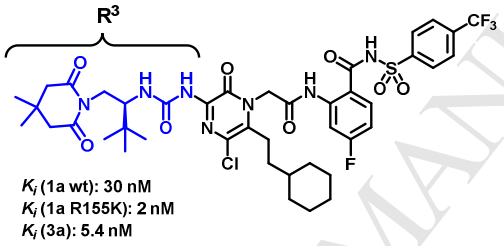
To create your abstract, type over the instructions in the template box below.
Fonts or abstract dimensions should not be changed or altered.

Pan-NS3 protease inhibitors of hepatitis C virus based on an R3-elongated pyrazinone scaffold

Leave this area blank for abstract info

Anna Karin Belfrage[a, *], Eldar Abdurakhmanov[b], Eva Åkerblom[a], Peter Brandt[a], Hiba Alogheli[a], Johan Neyts[c], U. Helena Danielson[b] and Anja Sandström[a]

[a]*Department of Medicinal Chemistry, Uppsala University, Box 574, SE-75123 Uppsala, Sweden.*
[b]*Department of Chemistry-BMC, Uppsala University, Box 576, SE-75123 Uppsala, Sweden.*
[c]*Rega Institute, Department of Microbiology and Immunology, University of Leuven, B-3000 Leuven, Belgium.*



K_i (1a wt): 30 nM
K_i (1a R155K): 2 nM
K_i (3a): 5.4 nM

European Journal of Medicinal Chemistry

Pan-NS3 protease inhibitors of hepatitis C virus based on an R3-elongated pyrazinone scaffold

Anna Karin Belfrage[a, *], Eldar Abdurakhmanov[b], Eva Åkerblom[a], Peter Brandt[a], Hiba Alogheli[a], Johan Neyts[c], U. Helena Danielson[b] and Anja Sandström[a]

[a]*Department of Medicinal Chemistry, Uppsala University, Box 574, SE-75123 Uppsala, Sweden.*
[b]*Department of Chemistry-BMC, Uppsala University, Box 576, SE-75123 Uppsala, Sweden.*
[c]*Rega Institute, Department of Microbiology and Immunology, University of Leuven, B-3000 Leuven, Belgium.*

ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keyword
Hepatitis C
NS3
Genotype 3
Resistance
Pyrazinone

ABSTRACT

Abstract Herein, we present the design and synthesis of 2(1*H*)-pyrazinone based HCV NS3 protease inhibitors and show that elongated R^3-urea substituents were associated with increased inhibitory potencies over several NS3 protein variants. The inhibitors are believed to rely on β-sheet mimicking hydrogen bonds which are similar over different genotypes and current drug resistant variants and correspond to the β-sheet interactions of the natural peptide substrate. Inhibitor **36**, for example, with a urea substituent including a cyclic imide showed balanced nanomolar inhibitory potencies against genotype 1a, both wild-type and R155K, and genotype 3a, K_i (1a) = 30 nM, K_i (R155K) = 2 nM and K_i (3a) = 5 nM).

1. Introduction

Approximately 71 million people worldwide are chronically infected with hepatitis C virus (HCV) with nearly 400 000 related deaths each year[1]. If left untreated, a chronic infection could lead to progression towards cirrhosis and hepatocellular carcinoma[2]. Besides host factors, e.g. age, gender and ethnicity, the genotype of the virus influences the rate of progression as well as the response to treatment[3]. Genotype (Gt) 3, the second most common genotype worldwide, is connected with greater risk of severe liver inflammation, faster progression of fibrosis and increased risk of hepatocellular carcinoma, compared to other genotypes[4]. Moreover, based on clinical and experimental data, Gt 3 is currently among the most difficult genotypes to treat[5,6]. Several drug discovery programs have focused on the development of direct-acting antivirals (DAAs) directed towards HCV infection[7]. After the first approved DAAs, i.e. the electrophilic boceprevir[8] (Victrelis™) and telaprevir[9] (Incivek™), the acidic product based NS3 protease inhibitor simeprevir[10] (Olysio™, **1**, Figure 1) and the NS5B polymerase inhibitor sofosbuvir[11] (Sovaldi™, **2**, Figure 1) entered the market in 2013. Subsequently, all-oral treatments, without the need for ribavirin or injection of pegIFNα, were approved[12–15]. These medications consist of combinations of two or three DAAs, e.g. Harvoni™ (ledipasvir[16], an NS5A inhibitor and **2**). Due to the error prone viral polymerase, a major challenge in anti-HCV drug discovery is the resistance-associated amino acid substitutions that emerge in the target protein under pressure of selective antiviral drugs[17,18]. For example, the amino acid substitutions R155K/T/Q confer resistance to all NS3 protease inhibitors approved and in clinical trials[19]. Considering the genotypic coverage of the NS3 protease inhibitors available on the market, they have limited efficacy against Gt 3 of the virus [20] but are highly effective against Gt 1[21,22]. However, grazoprevir (**3**), included in Zepatier™[14], shows balanced potency against Gt 1 and 3[23,24] and inspired development of promising, next-generation inhibitors with broad genotype potency[25]. While the majority of NS3 protease inhibitors evaluated in clinical trials or being approved show structural similarities to the discontinued drug ciluprevir (**4**), we have focused on the development of alternative classes to avoid the cross-resistance observed between the clinical candidates, resulting in novel 2(1*H*)-pyrazinone-based HCV NS3 protease inhibitors, exemplified by lead compound **5** (Figure 2)[26,27].

Herein, we present the design, synthesis and biochemical evaluation of 2(1*H*)-pyrazinone based inhibitors with elongated R^3-ureas and different acyl sulfonamide containing substituents against Gt 1a, both wild-type and the drug resistant enzyme variant R155K, as well as against Gt 3a of the virus. Selected inhibitors were evaluated in a cell based replicon assay.

* Corresponding author. Tel.: +46-184714337; e-mail: annakarin.belfrage@orgfarm.uu.se

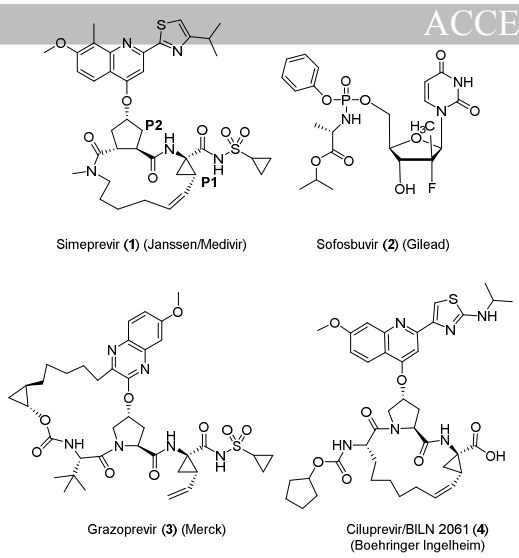


Figure 1. HCV NS3/4a protease inhibitors; **1**, **3** and **4** and the NS5B polymerase inhibitor **2**.

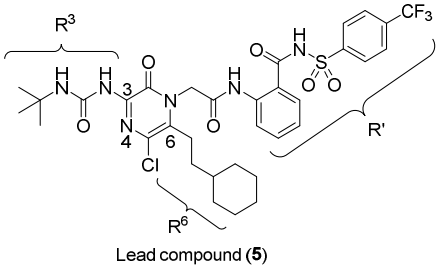


Figure 2. The lead compound **5**[27]; 2(1*H*)-pyrazinone-based HCV NS3 protease inhibitor, comprising an R' aromatic acyl sulfonamide, a *tert*-butyl urea in the R^3 position and an R^6-cyclohexyl ethyl group.

2. Chemistry

The syntheses of 2(1*H*)-pyrazinones with various elongated R^3-ureas and with different acyl sulfonamide containing R'-amides at N1 (Figure 3) started from preformed 6-benzyl- or 6-cyclohexyl-3-chloropyrazinones [27,28], followed by introduction of the ureas using two alternative methods: Insertion of an amine in R^3-position followed by reaction with an isocyanate (section 2.1), or a Pd-catalyzed C-N arylation[29] directly using presynthesized ureas (section 2.2-2.3). The inhibitors were finalized via amide coupling (section 2.4).

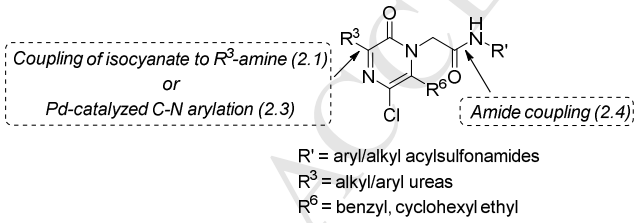
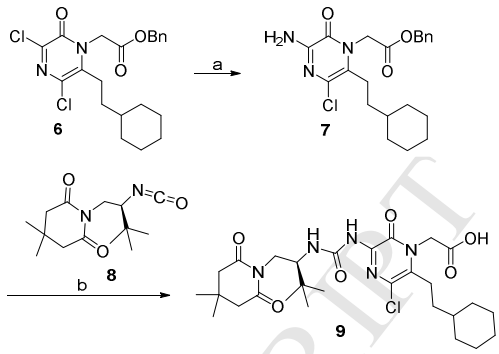


Figure 3. Schematic overview of key reactions to obtain 2(1*H*)-pyrazinone-based inhibitors with variations in R', R^3 and R^6.

2.1. Synthesis of R^3-urea substituted pyrazinone via isocyanate coupling to R^3-amine

The amine functionality in position 3 of the pyrazinone **7** was installed by addition of 25% NH_3 in H_2O to **6**[27] at 110 °C for 6 h (55%) (Scheme 1). The amino group in **7** was weakly nucleophilic, and required NaH as base for the subsequent installation of the urea functionality by employing the isocyanate **8**[30,31]. The benzyl ester on **7** and the 4,4-dimethylpiperidine-2,6-dione moiety on **9** hydrolyzed to some extent (THF/DME, 100 °C, 15-30 min) yielding a complicated reaction mixture. However, it was possible to isolate 43% of **9** after microwave heating at 100 °C for 15 min.

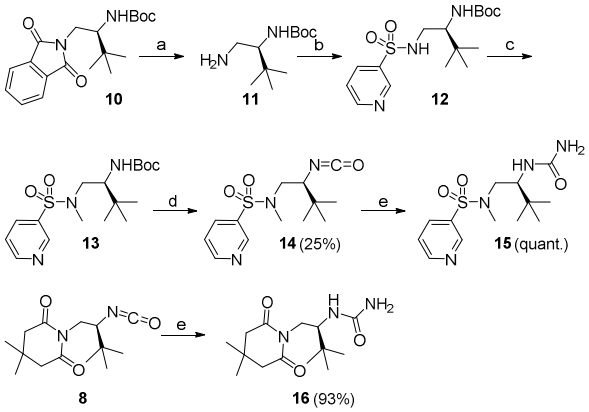


Scheme 1. Reagents and conditions: (a) 25% NH_3 in H_2O, 110 °C, 55%; (b) 60% NaH, DME, rt, MW 100 °C, 15 min, 43%.

2.2. Synthesis of urea building blocks

Presynthesized urea building blocks were prepared as outlined in Scheme 2. The phthalimido group of **10**[32,33] was deprotected with 40% methylamine in water and the resulting amine **11** was treated with 3-pyridine sulfonyl chloride to give the sulfonamide **12** in 66% yield. Alkylation of the nitrogen, to prevent intramolecular cyclization to a sulfonurea during the next coming triphosgene treatment (see supporting information, S2), using methyl iodide and cesium carbonate gave **13**. Subsequent Boc deprotection followed by treatment with triphosgene gave the isocyanate **14**[33]. The reaction also produced a major byproduct, the symmetric urea of two isocyanates **14**, which possibly is catalyzed by the pyridine moiety in **13** since a symmetric urea was not formed during the preparation of **8**[30,31] where no pyridine (or any tertiary amine)[34] was present.

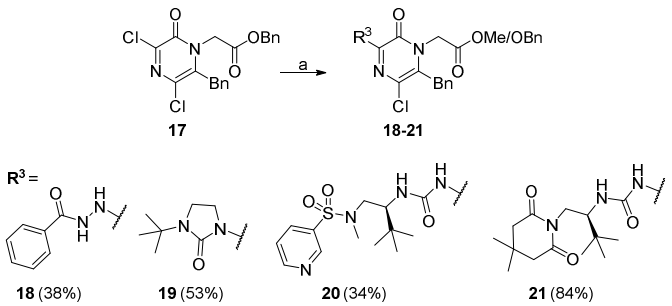
Finally, addition of 0.5 M ammonia in dioxane to **14** gave the urea derivative **15** in quantitative yield and the same reaction conditions starting with **8**[30,31] gave **16**[29] in 93% yield.



Scheme 2. Reagents and conditions: (a) 40% CH_3NH_2 in H_2O, rt, 88%; (b) 3-pyridine sulfonyl chloride, TEA, DCM, 0 °C/rt, 66%; (c) Cs_2CO_3, CH_3I, DMF, 0 °C/rt, 76%; (d) i. 4 M HCl in dioxane, rt; ii. sat. $NaHCO_3$ aq., DCM, triphosgene, 0 °C/rt; (e) 0.5 M NH_3 in dioxane, 0 °C/rt.;

2.3. Pd-catalyzed C-N arylation of 3-chloro-2(1H)-pyrazinones

The urea substituted pyrazinones **18**-**20** and **21**[29] were prepared via palladium catalyzed N-arylation of pyrazinone **17**[27,28] using benzohydrazide, 1-(*tert*-butyl)imidazolidin-2-one, **15**, or **16**[29]**,** respectively (Scheme 3). It was found that the benzyl ester was partly or fully converted into the methyl ester during the Pd catalyzed coupling reaction due to residual methanol in DME. Exchange of solvent to THF in the preparation of **18** resulted in a conserved benzyl ester. Anyhow, both ester derivatives were useful after final hydrolysis to yield the desired carboxylic acid as described in Scheme 4.

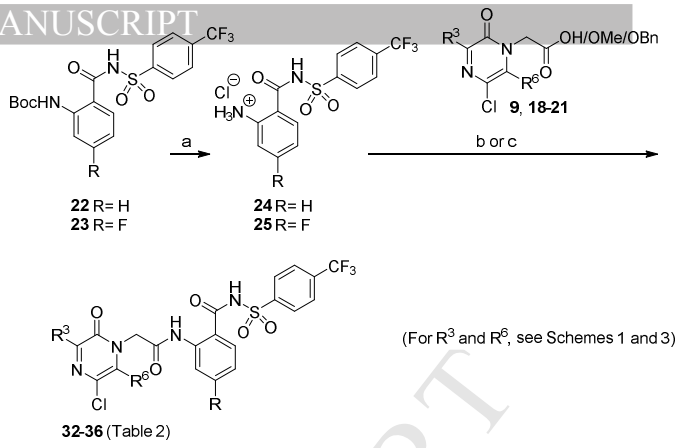


Scheme 3. Reagents and conditions: (a) Benzohydrazide, 1-(*tert*-butyl)imidazolidin-2-one, **15** or **16**, $Pd(OAc)_2$, Xantphos, Cs_2CO_3, DME or THF, MW 100 °C, 15-20 min.

*2.4. Amide coupling of R' substituents to the 2(1H)-pyrazinone containing carboxylic acid: Final inhibitors **5**, **26**-**36**.*

Boc deprotection of the previously described acylsulfonamide containing anilines **22** and **23** [26,35] yielded the R'-amines **24** and **25** (Scheme 4). The target compounds **32**-**34** were thereafter achieved after hydrolysis of the benzyl/methyl esters (**18**-**21**) followed by amide coupling with **24** and **25** by using $POCl_3$ (**32**, 8% and **33**, 11% over 2 steps) or *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridine-1-yl-methylene]-*N*-methylmethanamineium hexafluorophosphate *N*-oxide (HATU) (**34**, 52% over 2 steps).

The carboxylic acid **9** was coupled with **25** using HATU as coupling reagent yielding inhibitor **36** in 43% yield (Scheme 4). The remaining final inhibitors **5** and **26**-**30** (Table 1) as well as **31** and **35** (Table 2) were prepared as previously described[27,35].



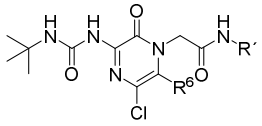
Scheme 4. Reagents and conditions: (a) 4 M HCl in dioxane, rt, quant; (b) **25** and **9**, HATU, DIEA, DCM, 45 °C; (c) i. **18**-**21**, K_2CO_3, CH_3CN/H_2O, MW 100 °C, 15 min; ii. **24** or **25**, $POCl_3$, pyridine, -15 °C/rt *or* HATU, DIEA, DCM, 45 °C.

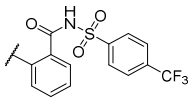
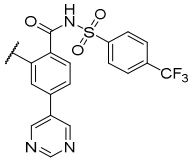
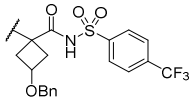
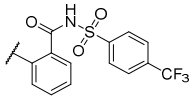
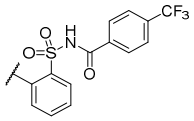
3. Biochemical and biological evaluation

Compounds **5** and **26**-**36** were biochemically evaluated in an *in vitro* assay using the full-length NS3 protein 1a and the central part of the NS4A as a cofactor (Tables 1-2)[36]. Ten inhibitors (**5**, **26**-**31**, **34**-**36**) were evaluated on the R155K mutant form of the protease ($NS3^{1a}{}_{flR155K}$) (Tables 1-2). The arginine (R) to lysine (K) mutation was introduced by PCR at position 155, and cloned in a similar way as has been described previously[37]. Eight compounds (**5**, **26**-**30**, **35** and **36**) were evaluated against the full-length NS3 Gt 3a (Tables 1-2).

Inhibition of viral replication (EC_{50}) was determined in a subgenomic HCV replicon assay[38] for compounds **27**[35] and **36** (Table 3).

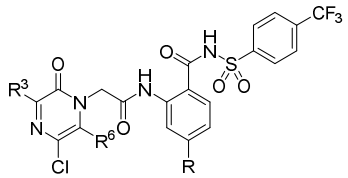
Table 1. NS3 inhibitory constants (K_i) of compounds **5**, **26-30**.

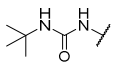
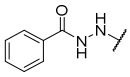
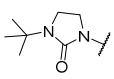
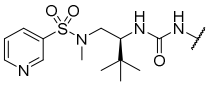
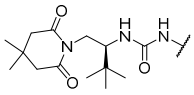
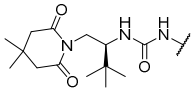


Compound	R´	R^6	$K_i \pm SD$ (nM)[b] 1a wt	$K_i \pm SD$ (nM)[b] 1a R155K	$K_i \pm SD$ (nM) 3a
5[a]			140 ± 20	30 ± 2	80 ± 40
26			120 ± 20	30 ± 4	87 ± 11
27			120 ± 30	17 ± 4	70 ± 5
28			70 ± 10	20 ± 3	68 ± 4
29[a]			310 ± 11	90 ± 10	565 ± 83
30			180 ± 30	30 ± 4	170 ± 14

[a]Compound previously reported in Gising et al.[27]; [b]Previously reported in Belfrage et al.[35]; SD, standard deviation.

Table 2. NS3 inhibitory constants (K_i) of compounds **31**-**36**.



Compound	R	R^3	R^6	$K_i \pm SD$ (nM) 1a wt	$K_i \pm SD$ (nM) 1a R155K	$K_i \pm SD$ (nM) 3a
31[a]	H			270 ± 80 [b]	70 ± 7 [b]	nd
32	H			1650 ± 46	nd	nd
33	H			380 ± 40	nd	nd
34	F			190 ± 40	40 ± 10	nd
35	F			160 ± 60	30 ± 5	135 ± 13
36	F			30 ± 2	2 ± 0.2	5.4 ± 0.4
4				0.089[c]	24[a]	20 ± 2
N-1725[d]				9 ± 2	nd	nd

[a]Previously reported in Gising et al.[27]; [b]Previously reported in Belfrage et al[35]; [c]Previously reported in Dahl et al.[37]; [d] Ac-Asp-D-Gla-Leu-Ile-Cha-Cys-OH; SD, standard deviation; nd, not determined.

Table 3. HCV replicon assay (Gt 1b): EC_{50} and CC_{50} values of inhibitors **27**, **36** and the reference compound **4**.

Compound	EC_{50}(μM)[a]	CC_{50}(μM)[b]
27	49[c]	>100[c]
36	7.1	26
4	0.002	>0.1

Mean value of two determinations.[a]50% effective concentration. [b]50% cytostatic/cytotoxic concentration. [c]Previously reported in Belfrage et al.[35].

4. Discussion

The design of the peptide mimicking pyrazinone-based HCV NS3 protease inhibitor series was initially based on the tripeptide **37** (see Figure 4, NS3 1a K_i = 5.4 μM)[39]. In this peptide, the *tert*-Leu-peptide part in P3 was replaced with a pyrazinone (i.e. a P3 pyrazinone) to yield an equipotent inhibitor **38** (NS3 1a K_i = 7.2 μM)[26]. Further, replacing the carbamate with a urea in R^3 and replacing the hydrophobic phenyl of the amino acid phenylglycine in P2 with a cyclohexyl ethyl in R^6 of the pyrazinone improved the inhibitory potency, exemplified by achiral peptidomimetic compound **5** (NS3 1a K_i = 140 nM, see Figure 4)[27].

Compound **5** and analogues thereof with modifications in the C-terminal acyl sulfonamide part, were previously evaluated as inhibitors of NS3 Gt 1a and the R155K variant showing a large acceptance for such structural variations with binding affinities in the sub-micromolar range (Table 1)[35]. Considering the need for inhibitors targeting difficult to treat enzyme variants such as Gt 3a, these inhibitors were further evaluated towards NS3 Gt 3a (data shown in Table 1, alongside their inhibition of the drug resistant Gt 1a variant R155K and Gt 1a wt). Thus, the K_i values (Gt 3a) differed less than ten-fold (i.e. 28, 68 nM and 29, 565 nM) and similar to the previous results from Gt 1a wt and R155K it was found that the C-terminal substituent had no significant effect on the inhibitory potency (e.g. **26**, 87 nM compared with **27**, 70 nM). The R^6 substituent, on the other hand, was shown to have certain impact on binding (**5**, 80 nM compared with **29**, 565 nM), as well as reversing the acyl sulfonamide functionality (**30**, 170 nM compared with **29**, 565 nM). However, in general, the structure activity relationship (SAR) for Gt 3a paralleled that of Gt 1a indicating similar binding interactions.

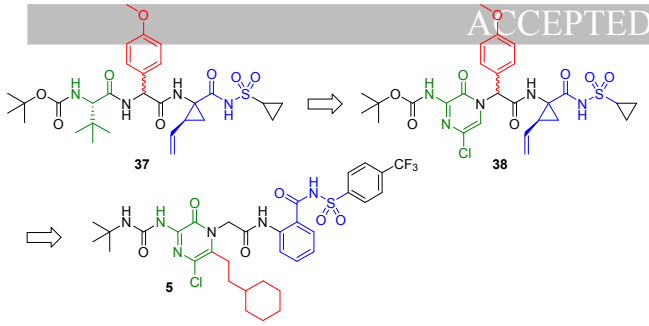


Figure 4. Tripeptide-based design of lead compound **5**.

Gallo and co-workers have characterized NS3 Gt 3a structurally by NMR spectroscopy[40]. They suggested that the lower activities towards Gt 3a as compared to Gt 1a observed for inhibitors with extended P2 substituents could be explained by differences in the corresponding binding pocket. In Gt 1a, salt bridges between the alternating acidic and basic side chains of R155, D168, and R123 are present, stabilizing side-chain conformations in favor of binding extended P2 substituents. The corresponding pocket in Gt 3a is constituted of the amino acids R155, Q168, and T123, lacking the salt bridges, thus being flexible in the apoenzyme. Upon binding inhibitors with large P2 substituents this side chain flexibility is prohibited and as a result, entropy will decrease and binding will be disfavored. The retained potency for inhibitors in the pyrazinone series towards Gt 3a could be rationalized by the lack of a large P2 group.

Alternatively, molecular dynamics simulations has suggested that different distances between the amino acids in the catalytic triad in Gt 3a compared with Gt 1a could explain the lower activity for highly optimized, product-based inhibitors[41]. In the pyrazinone series, the acidic acyl sulfonamide functionality in the C-terminal [26,27] has shown to have limited influence on inhibitory potency as discussed above[35]. Thus, the lack of tight S1 interactions could explain the retained inhibitory potency between Gt 1a and 3a for these compounds.

In 2011, Schiering *et al.* published a crystal structure of an HCV NS3 protease inhibitor in presence of both the NS3 protease and the helicase (PDB ID 4A92), displaying interactions from a P4-capping group with the helicase domain (His528)[42]. This result is in line with the P4 backbone interactions shown from the NS3 C-terminal part in the crystal structure of the full-length scNS3-NS4A from Yao *et al.* in 1999 (PDB ID 1CU1)[43]. These studies support previous results presented by our group showing the importance of using the full-length enzyme in the evaluation of inhibitors and for resistance profiling[37]. With a central interest in developing inhibitors with balanced potencies against drug-resistant variants of the enzyme, as well as against different genotypes, the strive for designing extended β-strand-based inhibitors that fit inside the natural substrate envelope was appealing[44,45].

Thus, instead of further elongating the R^6 position of the pyrazinone—which, according to an early binding hypothesis (see mode A in Figure 5) is expected to be directed towards the S2 pocket[27]—or to perform additional variations in the R' position (see Table 1), we decided to extended the inhibitors from the R^3-position of the pyrazinone (Table 2), aiming for additional interactions with the enzyme, mimicking the natural substrate. The N-terminal substituent 1-(4,4-dimethyl-2,6-di-oxopiperidin-1-yl)-3,3-dimethylbutan-2-ylurea **21** has previously been evaluated as a P3 capping group in an electrophilic HCV NS3 protease inhibitor[30]. Indeed, docking of inhibitor **36** being extended with **21** in the N-terminal[26], suggested additional hydrogen bond possibilities between the 4,4-dimethylpiperidine-2,6-dione oxygens and both His528 in the helicase and the backbone NH of Thr160 in the protease (see Figure 6). In addition, the interactions with the backbone (Lys136, Ala157 and Cys159) remained intact in this suggested binding mode (Binding mode A, Figure 5A and 6). To examine this hypothesis, we decided to synthesize and evaluate a few inhibitors extended in the R^3 position with groups of varying length, against NS3 Gt 1a wt and R155K, and 3a wt, according to Table 2.

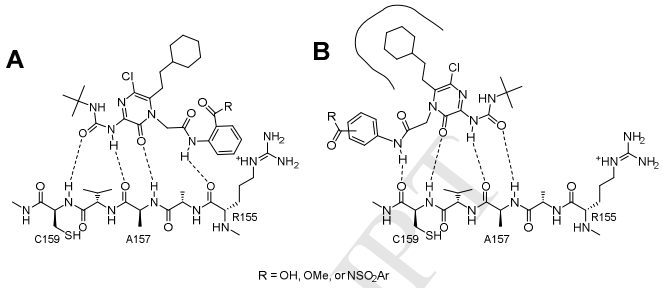


Figure 5. The two previously proposed binding modes evaluated for the current extended series. Mode A[26] places the acidic functionality juxtapose with the catalytic serine whereas in mode B[35] this moiety is water exposed and the hydrophobic R^6 substituent is buried in the helicase illustrated by the solid line.

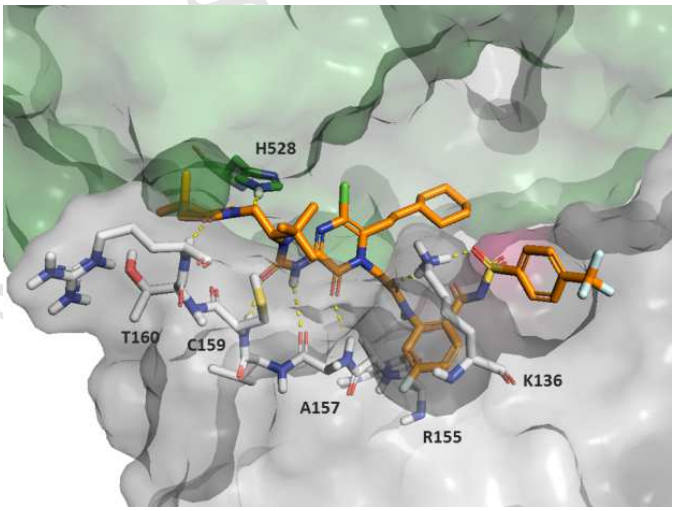


Figure 6. Binding mode A of inhibitor **36** (orange) modeled in the 4A92 crystal structure consisting of both the protease (grey surface) and the helicase (green surface) part. The hydrogen bond interactions are shown as dashed yellow lines. The catalytic triad is highlighted in pink. Hydrogen bonds are shown as dashed yellow lines.

Initially, inhibitors with two smaller R^3-capping groups were evaluated against Gt 1a (wt). Compound **32** with a phenyl hydrazide group replacing the urea, moving the carbonyl one step, showed a six times decreased inhibitory potency (1650 nM) compared with the urea **31** (270 nM). The decreased activity of **32** compared to **31** can be a result of several concurrent factors. For example, in **32**, an internal hydrogen bond to the pyrazinone is lost potentially increasing the desolvation energy. Further, a shift in the positioning of the hydrophobic tail group (Ph vs. *t*-Bu), or a less good interaction with the backbone NH of Cys159 could rationalize the reduced activity. However, compound **33**, a cyclized analogue of **31** lacking the free NH for hydrogen bonding to Ala157, retained potency (380 nM).

Next, three inhibitors with more extended R^3 substituents were evaluated. For the inhibitors with a benzyl in the R^6 position, the potency against Gt 1a was preserved (**34**, 190 nM and **35**, 160 nM compared with **31**, 270 nM). Gratifyingly, altering to a R^6-cyclohexyl ethyl group increased the potency 5-fold (**36**, 30 nM

compared with **35**, 160 nM) and more than 4-fold as compared to the corresponding inhibitor containing a *tert*-butyl urea as a P3-capping group (**5**, 140 nM). Yet more encouraging was the inhibitory potency against the R155K variant of the enzyme. Hence, compound **36** showed a K_i value of 2 nM, a 15-fold improvement to compound **35** (30 nM) and **5** (30 nM). Finally, the optimization was found valid also against the Gt 3a, where the inhibitory potency of **36** improved to 5.4 nM—25-fold and 15-fold better than **35** (135 nM) and **5** (80 nM), respectively.

Two compounds in the series (**27** and **368**) as well as the reference inhibitor **4** were evaluated in a cell-based replicon assay of Gt 1 (Table 3). Inhibitor **36** (7.1 µM), with a cyclic imide in the N-terminal and an aromatic acyl sulfonamide in the C-terminal showed slightly more promising cell-based data compared with the smaller sized compound **27** (49 µM), containing a *tert*-butyl urea in the N-terminal and a carboxylic acid in the C-terminal. The increased lipophilic character from the aromatic acyl sulfonamide compared with the truncated carboxylic acid could influence cell-permeability positively and thus improve the cell-based inhibitory potency, which has been observed previously for this series[35]. The drop in potency compared with the biochemical inhibition was expected[35,46] and also seen for the reference inhibitor **4** (K_i = 0.089 nM EC_{50} =0.002 µM).

Given the retained activity of the cyclic urea **33**, and previous observations[35] that C-terminal *o*-, *m*-, and *p*-amino benzoates are equipotent, binding mode A has been disputed. Therefore, alternative binding modes were investigated and a proposal more compatible with the SAR was suggested (see Figure 5B and Figure 7)[35]. In this binding mode, the more advanced R^3 tails such as in **36** may be less embraced by the protein and will be significantly water exposed whereas the R^6-cyclohexyl ethyl substituent occupies the hydrophobic pocket formed in the helicase. The corresponding hydrophobic interactions are not reached by compounds with a benzyl in the R^6 position. Instead the benzyl is pointing out of the pocket and a slight shift in the interaction with the beta strand allows binding of the R^3 substituent in the S1 pocket (see supporting information, Figure S1, compound **35**). The new binding mode B does not explain the retained activity of the cyclic urea (see supporting information, Figure S2, compound **33**). However, the shallow ligand binding pocket of the NS3 protease makes predictive docking challenging and a topic for future advances in molecular modeling.

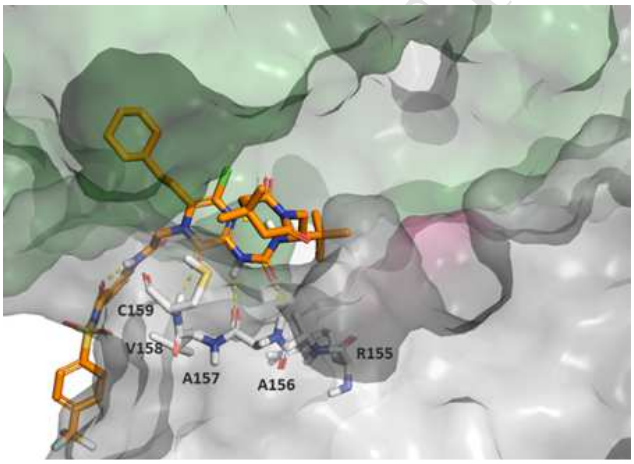


Figure 7. Binding mode B of inhibitor **36** (orange) modeled in the 4A92 crystal structure consisting of both the protease (grey) and the helicase (green) part. The hydrogen bond interactions are shown as dashed yellow lines. The catalytic triad is highlighted in pink. The R^6-cyclohexyl ethyl group is nicely occupying the hydrophobic S4 pocket and forming hydrogen bond interactions (dashed yellow lines) with C159, V158 and A156.

5. Conclusion

Results herein show that pyrazinone-based HCV NS3 protease inhibitors can be improved further by elongations in the N-terminal R^3 direction for several NS3 variants such as Gt 1a wt and the drug resistant variant R155K, and Gt 3a. For HCV NS3 Gt 1a, the improvement was close to five times whereas for the R155K mutant as well as for Gt 3a the improvement was fifteen times. The improved outcome in the two latter cases is probably attributed to limited interactions in the S2 pocket, in contrast to the highly affected and tight fitting clinical candidates with large P2 substituents. The overall SAR for pyrazinone-based inhibitors suggests a less tight binding mode relying on hydrogen bonds corresponding to the β-sheet interactions of the natural peptide substrate, and that are similar over different genotypes and current drug resistant variants. The well-balanced inhibitory potencies against the wild type and the drug resistant enzyme variant R155K of Gt 1a as well as against the Gt 3a of the virus, support the utility of this class of NS3 protease inhibitors in the development of a new generation NS3 protease inhibitors with a broad genotypic coverage.

6. Experimental section

6.1. Chemistry

NMR spectra were recorded on a Varian Mercury Plus at 25 °C for ^{1}H at 399.9 MHz and for ^{13}C NMR at 100.5 MHz. All microwave-assisted syntheses were carried out in a Smith synthesizer performed in sealed vials dedicated for microwave processing. Column flash chromatography was performed using silica gel 60 (particle size 0.040-0.063 mm, Sigma-Aldrich). Thin-layer chromatography was performed with aluminum sheets coated with silica gel 60- F_{254} (0.2 mm E. Merck), using UV-light for visualization. Analytical HPLC-UV/MS was performed on a Dionex UltiMate 3000 HPLC system with a Bruker amaZon SL ion trap mass spectrometer and detection by UV (DAD) and MS (ESI+), using a Phenomenex Kinetex C18 column (50×3.0 mm, 2.6 µM particle size, 100 Å pore size) and a flow rate of 1.5 mL/min. A gradient of H_2O/CH_3CN/0.05% HCOOH was used. Preparative RP-HPLC was performed by UV-triggered (254 nM) fraction collection with a Gilson HPLC system using an Agilent PrepHT Zorbax SB C8 column (150×21.2 mm, 5 µM particle size) and a H_2O/CH_3CN/0.05% HCOOH gradient at a flow rate of 10 mL/min. High resolution molecular mass (HRMS) was determined on a Micromass Q-Tof2 mass spectrometer equipped with an electrospray ion source. All products were >95% pure according to HPLC-UV at 254 nM. Optical rotation was obtained on a Rudolph Research Analytical Autopol II Automatic Polarimeter and the concentration *c* is given as g/100 ml in the specified solvent. Reactants and reagents that were commercially available were used without further purification. The synthesis of compounds **5**[27], **6**[27], **8**[29–31], **10**[32,33], **16**[29], **17**[27,28], **21**[29], **22**[26], **23**[29], **24**[26], **25**[29], **26-28**[35], **29**[27], **30**[35], **31**[27] and **35**[29] have been described elsewhere. Compounds **7**, **9**, **11-15**, **18-20**, **32-34** and **36** are new compounds which have been fully characterized.

6.1.1. Benzyl 2-(3-amino-5-chloro-6-(2-cyclohexylethyl)-2-oxopyrazin-1(2H)-yl)acetate (7)

6 (0.363 g, 0.86 mmol) was dissolved in CH_3CN (13 ml). 25% NH_3 in water (1.836 ml, 0.027 mol) was added and the reaction mixture was heated to 110 °C for 6 h. The solvent was evaporated followed by purification on silica gel (DCM/MeOH 95:5) to give the title compound in 55% yield (149 mg). ^{1}H NMR (CD_3OD) δ 7.37 – 7.34 (m, 4H), 5.23 (s, 2H), 4.81 (s, 2H), 2.54

(m, 2H), 1.72 – 1.61 (m, 5H), 1.36 – 1.12 (m, 6H), 0.88 (m, 2H). ^{13}C NMR (CD_3OD) δ 168.6, 152.7, 150.9, 136.6, 129.55, 129.49, 129.4, 125.7, 125.4, 68.5, 47.3, 38.6, 36.3, 33.9, 27.7, 27.4, 27.2 MS calcd for $C_{21}H_{26}ClN_3O_3$ [M+H$^+$]: 404.2 found: 404.2.

*6.1.2. (S)-2-(5-chloro-6-(2-cyclohexylethyl)-3-(3-(1-(4,4-dimethyl-2,6-dioxopiperidin-1-yl)-3,3-dimethylbutan-2-yl)ureido)-2-oxopyrazin-1(2H)-yl)acetic acid (**9**)*

7 (80 mg, 0.20 mmol) was dissolved in DME (2.8 ml), followed by addition of 60% NaH (0.024 g, 0.591 mmol). The reaction mixture was allowed to stir at rt for 5 min. **8** (0.105 g, 0.394 mmol) dissolved in DME (2 ml) was added and the reaction mixture was heated to 100 °C in the MW for 15 min. The resulting crude material was filtered and evaporated, followed by purification on silica gel (*iso*-hexane/EtOAc/HCOOH 50:50:3) to give the title compound in 43% yield (49 mg). ^{1}H NMR ($CDCl_3$) δ 4.70 (d, *J* = 16.4 Hz, 1H), 4.63 (d, *J* = 16.4 Hz, 1H), 4.09 (d, *J* = 11.9 Hz, 1H), 3.99 (t, *J* = 10.9 Hz, 1H), 3.90 (d, *J* = 12.0 Hz, 1H), 2.66 (t, *J* = 8.2 Hz, 2H), 2.45 (s, 4H), 1.79 – 1.65 (m, 5H), 1.44 – 1.10 (m, 6H), 1.05 (s, 9H), 1.01 (s, 6H), 1.10 – 0.90 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 172.8, 169.0, 154.9, 150.7, 143.4, 130.7, 122.4, 57.1, 47.1, 46.3, 40.0, 37.7, 35.1, 34.1, 33.0, 29.1, 27.8, 27.4, 26.6, 26.5, 26.3. MS calcd for $C_{28}H_{42}ClN_5O_6$ [M+H$^+$]: 580.3 found: 580.3.

*6.1.3. tert-Butyl (S)-(1-amino-3,3-dimethylbutan-2-yl)carbamate (**11**)*[32,33]

To **10** (1.69 g, 0,0053 mmol), methylamine (40% in H_2O) (17 ml, 0.219 mmol) was added and the reaction mixture was allowed to stir at rt overnight. The crude mixture was extracted with diethyl ether (2×20 ml) and the combined organic layers were evaporated to yield the title compound in 88% yield (1.01 g). ^{1}H NMR ($CDCl_3$) δ 4.47 (m, NH), 3.35 (td, *J* = 10.4, 3.2 Hz, 1H), 2.97 (m, 1H), 2.42 (dd, *J* = 13.3, 10.4 Hz, 1H), 1.94 (s, 2H), 1.44 (s, 9H), 0.90 (s, 9H). MS calcd for $C_{11}H_{24}N_2O_2$ [M+H$^+$]: 217.2 found: 217.0.

*6.1.4. tert-Butyl (S)-(3,3-dimethyl-1-(pyridine-3-sulfonamido)butan-2-yl)carbamate (**12**)*

3-pyridine sulfonylchloride (0.171 ml, 1.43 mmol) was dissolved in DCM (13 ml). **11** (0.205 g, 0.95 mmol) was added and the mixture was placed on an ice bath. TEA (0.530 ml, 3.8 mmol) was added and the reaction mixture was allowed to stir at the same temperature for 10 min, followed by stirring at rt for 2 h. The material was washed with 0.1 N $NaHSO_4$, evaporated and purified on silica gel (DCM/MeOH 95:65) to yield 226 mg of the title compound (66 %). ^{1}H NMR (CD_3OD) δ 8.97 (m, 1H), 8.76 (dd, *J* = 4.9, 1.6 Hz, 1H), 8.24 (ddd, *J* = 8.1, 2.4, 1.6 Hz, 1H), 7.62 (ddd, *J* = 8.1, 4.9, 0.9 Hz, 1H), 3.36 (td, *J* = 10.3, 3.0 Hz, 1H), 3.19 (dd, *J* = 13.4, 3.0 Hz, 1H), 2.80 (dd, *J* = 13.3, 10.4 Hz, 1H), 1.43 (s, 9H), 0.86 (s, 9H). ^{13}C NMR ($CDCl_3$) δ 155.2, 154.3, 148.5, 136.3, 135.0, 123.8, 58.3, 46.0, 33.7, 24.8. MS calcd for $C_{16}H_{27}N_3O_4S$ [M+H$^+$]: 358.2 found: 358.0.

*6.1.5. tert-Butyl (S)-(3,3-dimethyl-1-(N-methylpyridine-3-sulfonamido)butan-2-yl)carbamate (**13**)*

12 (0.345 g, 0.97 mmol) was dissolved in DMF (13 ml). Cs_2CO_3 (0.474 g, 1.46 mmol) was added and the mixture was placed on an ice bath under N_2 atm. Methyl iodide (0.413 g, 2.91 mmol) was added dropwise, and the reaction mixture was allowed to stir at the same temperature for 5 min followed by stirring at rt for 2 h. H_2O (5 ml) was added and the water layer was extracted with EtOAc (20 ml). The organic layer was further washed with H_2O (5 ml) and brine (5 ml), and evaporated to yield the title compound in 273 mg (76%). ^{1}H NMR ($CDCl_3$) δ 9.00 (dd, *J* = 2.3, 0.8 Hz, 1H), 8.80 (dd, *J* = 4.8, 1.6 Hz, 1H), 8.06 (ddd, *J* = 8.0, 2.4, 1.6 Hz, 1H), 7.47 (ddd, *J* = 8.0, 4.9, 0.9 Hz, 1H), 4.54 (m, NH), 3.67 (ddd, *J* = 11.4, 10.4, 3.7 Hz, 1H), 3.39 (dd, *J* = 13.6, 11.4 Hz, 1H), 2.85 (m, 1H), 2.81 (s, 3H), 1.46 (s, 9H), 0.92 (s, 9H). ^{13}C NMR ($CDCl_3$) δ 156.7, 153.3, 148.1, 135.4, 134.9, 123.9, 79.6, 55.6, 50.1, 34.7, 34.1, 28.5, 26.6. MS calcd for $C_{17}H_{29}N_3O_4S$ [M+H$^+$]: 372.2 found: 372.1.

*6.1.6. (S)-N-(2-isocyanateo-3,3-dimethylbutyl)-N-methylpyridine-3-sulfonamid (**14**)*

13 (0.262 mg, 0.705 mmol) was dissolved in 4 M HCl in dioxane (10 ml) and allowed to stir at rt for 3 h. The solvent was evaporated and the residue was dissolved in DCM (8 ml) and sat. $NaHCO_3$ (aq) (8 ml) and placed on an ice bath. Triphosgene (0.259 g, 0.871 mmol) was added and the reaction mixture was stirred vigourously at the same temperature for 15 min followed by stirring at rt for 4 h. Extraction with DCM (8 ml) and the organic layer was evaporated and purified on silica gel ((*iso*-hexane/EtOAc 1:3). The title compound was obtained in 25 % yield (52 mg). ^{1}H NMR ($CDCl_3$) δ 9.03 (m, 1H), 8.83 (m, 1H), 8.10 (m, 1H), 7.51 (ddt, *J* = 8.1, 4.9, 0.9 Hz, 1H), 3.58 (m, 1H), 3.22 (dd, *J* = 13.8, 2.8 Hz, 1H), 3.08 (dd, *J* = 13.8, 10.5 Hz, 1H), 2.89 (s, 3H), 0.96 (s, 9H). ^{13}C NMR ($CDCl_3$) δ 153.6, 148.2, 135.2, 134.4, 124.0, 64.6, 52.2, 36.5, 34.5, 28.5. MS calcd for $C_{13}H_{19}N_3O_3S$ [M+H$^+$]: 298.1 found: 298.1.

*6.1.7. (S)-N-(3,3-dimethyl-2-ureidobutyl)-N-methylpyridine-3-sulfonamid (**15**)*

14 (52 mg, 0.174 mmol) was dissolved in DCM (6 ml) and placed on an ice bath. 0.5 M NH_3 in dioxane (1.04 ml, 0.521 mmol) was added dropwise and the reaction mixture was allowed to stir at rt for 3 h. The solvent was evaporated and the urea **15** was not further purified (55 mg, quant.). ^{1}H NMR ($CDCl_3$) δ 9.00 (m, 1H), 8.80 (dd, *J* = 4.9, 1.6 Hz, 1H), 8.07 (m, 1H), 7.48 (ddd, *J* = 8.0, 4.8, 0.8 Hz, 1H), 5.18 (m, NH), 4.76 (brs, NH), 3.85 (m, 1H), 3.47 (m, 1H), 2.83 (s, 3H), 0.91 (s, 9H). ^{13}C NMR ($CDCl_3$) δ 159.5, 153.3, 148.0, 135.3, 135.1, 124.0, 54.5, 50.4, 34.8, 34.2, 26.6. MS calcd for $C_{13}H_{22}N_4O_3S$ [M+H$^+$]: 315.1 found: 315.2.

*6.1.8. General procedure for coupling of 2(1H)-pyrazinone and urea (**18-20**). Method A.*

A 5 ml microwave vial was charged with **17** (1 equiv.), urea equivalent (3 equiv.), $Pd(OAc)_2$ (0.08 equiv.), Xantphos (0.10 equiv.) and Cs_2CO_3 (2 equiv.) in dry DME or THF (2-3 ml). The vial was capped under air and irradiated by MW to 100 °C for 15-20 min. Purified as stated yielding compound **18-20** in 38-53% yield.

*6.1.9. Benzyl 2-(3-(2-benzoylhydrazinyl)-6-benzyl-5-chloro-2-oxopyrazin-1(2H)-yl)acetate (**18**)*

The title compound was prepared as described in general method A: **17** (75 mg, 0.186 mmol), benzohydrazide (38 mg, 0.279 mmol), $Pd(OAc)_2$ (3 mg, 0.013 mmol), Xantphos (11 mg, 0.019 mmol) and Cs_2CO_3 (121 mg, 0.372 mmol) in THF (3 ml) at 100 °C for 15 min. The crude material was filtered and the solvent was evaporated. Purification by silica column chromatography, eluent *iso*-hexane/EtOAc 1:1 and a second purification, eluent *iso*-hexane/EtOAc/HCOOH 4:1:0.15, gave the compound in 38% yield (36 mg). ^{1}H NMR ($CDCl_3$) δ 7.89 (m, 2H), 7.52 (m, 1H), 7.44 (m, 2H), 7.37 – 7.17 (m, 8H), 7.03 (m, 2H), 5.04 (s, 2H), 4.60 (s, 2H), 3.95 (s, 2H). ^{13}C NMR ($CDCl_3$) δ 166.61, 165.88, 150.60, 146.64, 135.19, 134.81, 132.43, 132.37, 131.81, 129.25, 128.74, 128.72, 128.40, 127.75, 127.53, 127.46, 126.42, 123.99, 67.81, 46.10, 35.25. HRMS (ES) *m/z* calcd for $C_{27}H_{23}ClN_4O_4$ [M+H$^+$]: 503.1486 found 503.1496.

*6.1.10. Benzyl 2-(6-benzyl-3-(3-(tert-butyl)-2-oxoimidazolidin-1-yl)-5-chloro-2-oxopyrazin-1(2H)-yl)acetate (**19**)*

The title compound was prepared as described in general method A: **17** (100 mg, 0.25 mmol), 1-(*tert*-butyl)imidazolidin-2-one (106 mg, 0.74 mmol), $Pd(OAc)_2$ (4 mg, 0.017 mmol), Xantphos (14 mg, 0.025 mmol) and Cs_2CO_3 (162 mg, 0.496 mmol) in DME (2 ml) at 100 °C for 15 min. The crude material was filtered and the solvent was evaporated. Purification by silica column chromatography, eluent *iso*-hexane/EtOAc 4:1-1:1 gave the compound in 53% yield (67 mg). ^{1}H NMR (CD_3OD) δ 7.38 – 7.23 (m, 8H), 7.16 (ddt, *J* = 7.3, 1.5, 0.7 Hz, 2H), 4.98 (s, 2H), 4.75 (s, 2H), 4.20 (s, 2H), 3.87 (m, 2H), 3.59 (m, 2H), 1.42 (s, 9H). ^{13}C NMR (CD_3OD) δ 167.71, 157.91, 153.83, 146.54, 136.67, 136.06, 134.65, 130.15, 129.58, 129.43, 129.25, 129.16, 128.47, 126.17, 68.43, 55.09, 44.12, 42.19, 36.25, 27.92, 24.41. HRMS (ES) *m/z* calcd for $C_{27}H_{29}ClN_4O_4$ [M+H$^+$]: 509.1956 found 509.1946.

*6.1.11 Benzyl (S)-2-(6-benzyl-5-chloro-3-(3-(3,3-dimethyl-1-(N-methylpyridine-3-sulfonamido)butan-2-yl)ureido)-2-oxopyrazin-1(2H)-yl)acetate (**20**)*

The title compound was prepared as described in general method A: **17** (59 mg, 0.146 mmol), **15** (69 mg, 0.219 mmol), $Pd(OAc)_2$ (2 mg, 0.007 mmol), Xantphos (7 mg, 0.012 mmol) and Cs_2CO_3 (95 mg, 0.293 mmol) in DME (3 ml) at 100 °C for 20 min. The crude material was filtered and the solvent was evaporated. Purification by silica column chromatography, eluent *iso*-hexane/EtOAc 4:1 – 100% EtOAc gave the compound in 34% yield (30 mg). ^{1}H NMR ($CDCl_3$) δ 8.98 (dd, *J* = 2.3, 1.0 Hz, 1H), 8.71 – 8.60 (m, 2H), 8.04 (m, 1H), 7.94 (s, NH), 7.38 (m, 1H), 7.32 (m, 2H), 7.28 (m, 1H), 7.18 (m, 2H), 4.64 (d, *J* = 7.6, 2H), 4.10 (s, 2H), 4.01 (m, 1H), 3.67 (s, 3H), 3.55 (dd, *J* = 13.8, 10.8 Hz, 1H), 3.15 (m, 1H), 2.96 (s, 3H), 1.04 (s, 9H). ^{13}C NMR (CDCl) δ 166.7, 153.1, 153.1, 150.8, 148.0, 144.3, 136.0, 134.8, 134.7, 129.4, 127.9, 127.8, 127.2, 124.2, 123.8, 55.5, 53.1, 50.9, 46.6, 35.5, 34.8, 34.1, 26.7. HRMS (ES) *m/z* calcd for $C_{33}H_{37}ClN_6O_6S$ [M+H$^+$]: 605.1943 found 605.1949.

*6.1.12. General procedure for preparation of the final inhibitors **32**-**34** and **36**. Method B.*

A 2-5 ml microwave vial was charged with the benzyl/methyl ester of the pyrazinone (**18**-**20**) K_2CO_3, CH_3CN, and H_2O. The vial was capped under air and irradiated by MW to 100-120 °C for 15-20 min[26]. 1 M HCl was added and the mixture was extracted with EtOAc. The organic layers were dried over $MgSO_4$ and evaporated.

Method B1: The crude acid and the amine (free or as the hydrochloride salt) were dissolved in pyridine and cooled to -15 °C under N_2. $POCl_3$ was added and the reaction mixture was stirred at -15 °C to room temperature for 0.5-2 days. In most of the cases, extra $POCl_3$ (reaction on ice/aceton bath) and/ or amine was added to consume the pyrazinone-carboxylic acid. H_2O (20 ml) was added and the pH adjusted to 1 using 6.0 M HCl aq. followed by extraction with EtOAc (2-3 x 20-30 ml). The organic layer was dried over $MgSO_4$ and evaporated. Purification by silica column flash chromatography gave the product.

Method B2: To the crude acid, the amine (hydrochloride salt) and HATU dissolved in dry DCM, DIEA was added and pH controlled to be 10 or higher. The reaction mixture was allowed to stir at 45 °C for 2-4.5 h. After addition of DCM and washing with 0.1 M $NaHSO_4$ aq. the organic layer was evaporated. Purification on silica column gave the final compound.

*6.1.13. General procedure for Boc deprotection of compounds **22** and **23** to yield **24** and **25***

The R´ building block was dissolved in 4 M HCl in dioxane and stirred at room temperature. When the reaction was complete (LC-MS analysis) the solvent was evaporated in vacuo. The resulting hydrochloride (**24** or **25**) was dried under vacuum and used in the subsequent coupling reactions without further purification.

*6.1.14. 2-(2-(3-(2-Benzoylhydrazinyl)-6-benzyl-5-chloro-2-oxopyrazin-1(2H)-yl)acetamido)-N-((4-(trifluoromethyl)phenyl)sulfonyl)benzamide (**32**)*

The title compound was prepared according to method B1. **18** (42 mg, 0.084 mmol), K_2CO_3 (17 mg, 0.125 mmol), CH_3CN (2 ml), H_2O (1 ml). Irradiated by MW to 100 °C for 15 min. 1 M HCl (20 ml), EtOAc (2×20 ml). The crude acid was dissolved in pyridine (2 ml). **24** (45 mg, 0.119 mmol) was added followed by $POCl_3$ (11 mg, 0.071 mmol), -15 °C, 10 min, rt, 2 h 45 min. *Extra additions of $POCl_3$:* after 45 min, 1 h 45 min: $POCl_3$ (5 mg, 0.032 mmol). H_2O (20 ml). Extracted with EtOAc (2×20 ml). Purification by preparative RP-HPLC, 25 mM NH_4OAc; CH_3CN/H_2O, 30-90%, 60 min. gave **32** in 8% yield, 5 mg as white solid. ^{1}H NMR (CD_3OD) δ 8.23 – 8.15 (m, 3H), 8.12 (dd, *J* = 8.0, 1.7 Hz, 1H), 8.00 (m, 2H), 7.81 (dm, *J* = 8.4 Hz, 2H), 7.59 (m, 1H), 7.51 (ddd, *J* = 8.4, 6.6, 1.5 Hz, 2H), 7.29 (ddd, *J* = 8.5, 7.3, 1.7 Hz, 1H), 7.14 – 6.94 (m, 6H), 4.73 (s, 2H), 4.16 (s, 2H). ^{13}C NMR (CD_3OD) δ 169.8, 165.9, 152.5, 151.7, 150.0, 148.9, 140.7, 137.0, 133.8, 133.7 (q, *J* = 32.2 Hz), 133.3, 132.8, 132.1, 129.8, 129.7, 129.3, 129.2, 128.8, 127.9, 127.8, 126.5 (q, *J* = 3.8 Hz), 126.0, 125.2 (q, *J* = 271.9 Hz), 124.3, 123.9, 120.9, 49.9, 35.9. HRMS calcd for $C_{34}H_{26}ClF_3N_6O_6S$ [M+H$^+$]: 739.1353; found 739.1354.

*6.1.16. 2-(2-(6-Benzyl-3-(3-(tert-butyl)-2-oxoimidazolidin-1-yl)-5-chloro-2-oxopyrazin-1(2H)-yl)acetamido)-N-((4-(trifluoromethyl)phenyl)sulfonyl)benzamide (**33**)*

The title compound was prepared according to method B1. **19** (33 mg, 0.065 mmol), K_2CO_3 (14 mg, 0.098 mmol), CH_3CN (2 ml), H_2O (1 ml). Irradiated by MW to 100 °C for 15 min. 1 M HCl (20 ml), EtOAc (2×20 ml). The crude acid was dissolved in pyridine (2 ml). **24** (37 mg, 0.097 mmol) was added followed by $POCl_3$ (11 mg, 0.071 mmol), -15 °C, 10 min, room temperature, 2 h. *Extra additions of $POCl_3$:* after 1 h, 1.5 h: $POCl_3$ (5 mg, 0.032 mmol). H_2O (20 ml). Extracted with EtOAc (2×20 ml). Purification by silica column flash chromatography (EtOAc/*i*-hexane/HCOOH 50:50:0.5) followed by a second column (DCM/MeOH 98:2) gave **33** in 11% yield, 5 mg as white solid. ^{1}H NMR (CD_3OD) δ 8.14 (dm, *J* = 8.2 Hz, 2H), 8.09 (dd, *J* = 8.1, 2.2 Hz, 2H), 7.83 (dm, *J* = 8.2 Hz, 2H), 7.33 (m, 1H), 7.15 – 6.92 (m, 6H), 4.76 (s, 2H), 4.28 (s, 2H), 4.01 (dd, *J* = 8.5, 7.0 Hz, 2H), 3.63 (m, 2H), 1.42 (s, 9H). HRMS calcd for $C_{34}H_{32}ClF_3N_6O_6S$ [M+H$^+$]: 745.1823; found 745.1825.

*6.1.16. (S)-2-(2-(6-benzyl-5-chloro-3-(3-(3,3-dimethyl-1-(N-methylpyridine-3-sulfonamido)butan-2-yl)ureido)-2-oxopyrazin-1(2H)-yl)acetamido)-4-fluoro-N-((4-(trifluoromethyl)phenyl)sulfonyl)benzamide (**34**)*

The title compound was prepared as described in method B2: **20** (0.030 g, 0.050 mmol), K_2CO_3 (0.010 g, 0.074 mmol), CH_3CN (2 ml), and H_2O (1 ml). 100 °C, 15 min. 1 M HCl (20 ml) EtOAc (2×20 ml). **25** (0.029 g, 0.074 mmol), DCM (2 ml), DIEA (0.051 ml, 0.294 mmol), HATU (0.022 g, 0.059 mmol). 45 °C for 3 h. DCM (15 ml), 0.1 N $NaHSO_4$ (20 ml). Purification on silica gel four times (*iso*-hexane/EtOAc/HCOOH 50:50:0.5×2, 100 % DCM – DCM/MeOH 92:8×2) gave the title compound in 52 % yield (24 mg). ^{1}H NMR (CD_3OD) 8.88 (dd, *J* = 2.3, 0.8 Hz, 1H), 8.70 (ddm, *J* = 4.9, 1.6 Hz, 1H), 8.36 – 8.05 (m, 5H), 7.78 (dm, *J* = 8.3 Hz, 2H), 7.57 (ddd, *J* = 8.1, 4.9, 0.8 Hz, 1H), 7.36 – 7.03 (m, 5H), 6.79 (m, 1H), 4.85 (s, 2H), 4.27 (m, 2H), 3.89 (m, 1H),

3.46 (m, 1H), 3.52 (m, 4H), 0.96 (s, 9H). ^{13}C NMR (CD_3OD) δ 173.0, 170.3, 166.0 (d, *J* = 248.7 Hz), 164.7, 160.4, 154.0, 152.3, 148.8, 148.4, 145.3, 142.7 (d, *J* = 13.6 Hz), 137.3, 136.6, 136.5, 135.3 (d, *J* = 11.3 Hz), 133.9 (d, *J* = 32.5 Hz), 129.9, 129.8, 129.3, 128.2, 128.0, 126.9 (d, *J* = 3.8 Hz), 125.8, 125.1 (d, *J* = 271.8 Hz), 121.0, 110.8 (d, *J* = 22.3 Hz), 107.7 (d, *J* = 30.8 Hz), 56.8, 51.7, 45.6, 36.0, 34.8, 29.1, 27.1. HRMS calcd for $C_{40}H_{39}ClF_4N_8O_8S_2$ [M+H$^+$]: 935.2052; found 935.2035.

*6.1.17. (S)-2-(2-(5-chloro-6-(2-cyclohexylethyl)-3-(3-(1-(4,4-dimethyl-2,6-dioxopiperidin-1-yl)-3,3-dimethylbutan-2-yl)ureido)-2-oxopyrazin-1(2H)-yl)acetamido)-4-fluoro-N-((4-(trifluoromethyl)phenyl)sulfonyl)benzamide (**36**)*

The title compound was prepared as described in general method B2, but without the hydrolysis of benzyl ester. **25** (0.023 g, 0.065 mmol), DCM (2 ml), DIEA (0.046 ml, 0.269 mmol), **9** (0.025 g, 0.042 mmol), HATU (0.019 g, 0.050 mmol). 45 °C for 4.5 h. DCM (12 ml), 0.1 N $NaHSO_4$ (8 ml). Purification on silica gel (*iso*-hexane/EtOAc/HCOOH 50:50:1, followed by DCM/MeOH 9:1) gave 28 mg of the title compound (71%). ^{1}H NMR (CD_3OD) δ 8.22 – 8.10 (brs, 2H), 8.19 (d, *J* = 7.9 Hz, 2H), 7.76 (d, *J* = 7.9 Hz, 2H), 6.79 – 6.64 (brs, 1H), 5.02 – 4.92 (brs, 1H), 4.86 – 4.78 (brs, 1H), 4.14 (dd, *J* = 11.9, 12.6 Hz, 1H), 4.04 (dd, *J* = 2.4, 11.8 Hz, 1H), 3.89 (dd, *J* = 2.5, 12.6 Hz, 1H), 2.79 – 2.65 (brs, 2H), 2.53 – 2.42 (brs, 4H), 1.74 – 1.50 (brs, 5H), 1.46 – 1.21 (brs, 4H), 1.21 – 0.98 (brs, 2H), 1.09 (s, 9H), 1.01 (s, 6H), 0.95 – 0.78 (brs, 2H). ^{13}C NMR (CD_3OD) δ 174.5, 173.5, 166.3, 166.0 (d, *J* = 248.7 Hz), 155.8, 152.2, 148.9, 144.7, 142.7 (d, *J* = 9.7 Hz), 134.7 (d, *J* = 9.9 Hz), 134.2 (q, *J* = 32.4 Hz), 132.7, 128.9, 126.6 (q, *J* = 3.7 Hz), 125.1 (q, *J* = 266.0 Hz), 123.2, 120.7, 110.6 (d, *J* = 22.8 Hz), 107.6 (d, *J* = 27.9 Hz), 58.0, 50.6, 46.9, 40.7, 38.7, 35.9, 35.1, 34.0, 29.8, 28.3, 27.8, 27.5, 27.2, 26.9. HRMS (ES) *m/z* calcd for $C_{42}H_{50}ClF_4N_7O_8S$ [M+H$^+$]: 924.3144; found 924.3139.

6.2 Enzyme inhibition

The protease activity of the full-length HCV NS3 protein (protease-helicase/NTPase) was measured using a FRET-assay as previously described[36,47]. In short, NS3 enzyme (1a wt: 0.25 nM, R155K: 0.08 nM, 3a: 0.25 nM) was incubated for 10 minutes at 30 °C in 50 mM HEPES, pH 7.5, 10 mM DTT, 40% glycerol, 0.1% n-octyl-*β*-D-glucoside, 3.3% DMSO with 25 µM of the peptide cofactor 2K-NS4A (KKGSVVIVGRIVLSGK) and inhibitor. The reaction was started by the addition of 0.5 µM substrate (Ac-DED(Edans)EEAbu*ψ*-[COO]ASK(Dabcyl)-NH2) obtained from AnaSpec Inc. (San Jose, USA). The non-linear regression analysis was made using Grafit 5.0.8 (Erithacus Software Limited).

Table 4. Kinetic parameters of tested enzyme variants.

Parameters	NS3 1a (WT)	NS3 R155K	NS3 3a
k_{cat} (s^{-1})	11.9±1.07	29.4±1.12	10.26±0.44
K_M (µM)	0.97±0.22	0.15±0.02	0.80±0.09

The compounds auto-fluorescence was corrected by measuring the fluorescence intensities at each tested concentrations. The percentage of correction was calculated by dividing the fluorescence intensity in presence of compound to fluorescence intensity in the absence of compound and applied to the enzyme's measured Initial velocities. The presence of 15 µM DABCYL did not affect the auto-fluorescence of the compounds meaning that no compounds auto-fluorescence quenching could be observed.

6.3 Modeling

All calculations were performed within the Schrödinger Small Molecule Drug Discovery Suite (Schrödinger Release 2016-4). The crystal structure (4A92) was downloaded from the PDB and prepared as described earlier[35]. The OPLS3 force field was used throughout this work[48], in combination with the GB/SA continuum solvation model for water[49]. Poses disclosed in an earlier publication [35] were used as starting points for subsequent molecular mechanics conformational searches in MacroModel (Schrödinger Release 2016-4: MacroModel, Schrödinger, LLC, New York, NY, 2016).

The conformational search of the compounds used 1000 MCMM search steps. A substructure of amino acid side-chains within 6 Å of the ligand were allowed full flexibility. A shell of constrained atoms (radius 6Å) around the substructure was defined with harmonic positional constraints with force constant of 200 kcal mol^{-1} Å^{-2}, and a shell of frozen atoms (radius 6Å) around the first shell was applied. The conformational search of compound **36** in pose B used three flat bottomed constraints with force constants of 100 kcal mol^{-1} Å^{-2}; one from pyrazinone carbonyl to the NH of C159, one from the NH attached to the pyrazinone to the carbonyl of A157, and one from urea carbonyl to the NH of A157. The distance constraints were set to 2.5 ± 0.5 Å. The final poses were relaxed without constraints and with full flexibility of the amino acids C159, V158, A157, and A156.

Acknowledgments

We acknowledge Gunnar Lindeberg for assistance in the purification of the co-factor, NS4A protein, used in the biochemical assay and the Swedish Research Council for financial support (#D0571301, HD).

Supplementary data

Supplementary data related to this article can be found at http://...

References and notes

[1] Hepatitis C Fact Sheet; World Health Organization (WHO); Updated April 2017, (n.d.) http://www.who.int/mediacentre/ factsheets/fs164/e.

[2] J.H. Hoofnagle, Hepatitis C : The clinical spectrum of disease, Hepatology. 26 (1997) 15S–20S.

[3] J.S. Doyle, M.E. Hellard, A.J. Thompson, The role of viral and host genetics in natural history and treatment of chronic HCV infection., Best Pract. Res. Clin. Gastroenterol. 26 (2012) 413–427. doi:10.1016/j.bpg.2012.09.004.

[4] F. Ansaldi, A. Orsi, L. Sticchi, B. Bruzzone, G. Icardi, Hepatitis C virus in the new era: perspectives in epidemiology, prevention, diagnostics and predictors of response to therapy., World J. Gastroenterol. 20 (2014) 9633–9652. doi:10.3748/wjg.v20.i29.9633.

[5] N. Goossens, F. Negro, Is genotype 3 of the hepatitis C virus the new villain?, Hepatology. 59 (2014) 2403–2412. doi:10.1002/hep.26905.

[6] E.B. Tapper, N.H. Afdhal, Is 3 the new 1: perspectives on virology, natural history and treatment for hepatitis C genotype 3., J. Viral Hepat. 20 (2013) 669–77. doi:10.1111/jvh.12168.

[7] A.D. Kwong, The HCV revolution did not happen overnight, ACS Med. Chem. Lett. 5 (2014) 214–220.

[8] B.A. Malcolm, R. Liu, F. Lahser, S. Agrawal, B. Belanger, R. Chase, F. Gheyas, A. Hart, D. Hesk, C. Jiang, R. Kong, J. Lu, J. Pichardo, A. Prongay, A. Skelton, X. Tong, S. Venkatraman, E. Xia, F.G. Njoroge, N. Butkiewicz, P. Ingravallo, V. Girijavallabhan, SCH 503034, a mechanism-based inhibitor of hepatitis C virus NS3 protease, suppresses polyprotein maturation and enhances the antiviral activity of alpha interferon in replicon cells, Antimicrob. Agents Chemother. 50 (2006) 1013–1020. doi:10.1128/AAC.50.3.1013.

[9] C. Lin, A.D. Kwong, R.B. Perni, Discovery and development of VX-950, a novel, covalent, and reversible inhibitor of hepatitis C Virus NS3 • 4A serine protease, Infect. Disord. - Drug Targets. 6 (2006) 3–16.

[10] Å. Rosenquist, B. Samuelsson, P.-O. Johansson, M.D. Cummings, O. Lenz, P. Raboisson, K. Simmen, S. Vendeville, H. de Kock, M. Nilsson, A. Horvath, R. Kalmeijer, G. de la Rosa, M. Beumont-Mauviel, Discovery and development of simeprevir (TMC435), a HCV NS3/4A protease inhibitor., J. Med. Chem. 57 (2014) 1673–1693. doi:10.1021/jm401507s.

[11] M.J. Sofia, D. Bao, W. Chang, J. Du, D. Nagarathnam, S. Rachakonda, P.G. Reddy, B.S. Ross, P. Wang, H.-R. Zhang, S. Bansal, C. Espiritu, M. Keilman, A.M. Lam, H.M.M. Steuer, C. Niu, M.J. Otto, P.A. Furman, Discovery of a β-d-2'-deoxy-2'-α-fluoro-2'-β-C-methyluridine nucleotide prodrug (PSI-7977) for the treatment of hepatitis C virus., J. Med. Chem. 53 (2010) 7202–7218. doi:10.1021/jm100863x.

[12] N. Afdhal, K.R. Reddy, D.R. Nelson, E. Lawitz, S.C. Gordon, E. Schiff, R. Nahass, R. Ghalib, N. Gitlin, R. Herring, J. Lalezari, Z.H. Younes, P.J. Pockros, A.M. Di Bisceglie, S. Arora, G.M. Subramanian, Y. Zhu, H. Dvory-Sobol, J.C. Yang, P.S. Pang, W.T. Symonds, J.G. McHutchison, A.J. Muir, M. Sulkowski, P. Kwo, Ledipasvir and sofosbuvir for previously treated HCV genotype 1 infection., N. Engl. J. Med. 370 (2014) 1483–1493. doi:10.1056/NEJMoa1316366.

[13] P. Andreone, M.G. Colombo, J. V. Enejosa, I. Koksal, P. Ferenci, A. Maieron, B. Müllhaupt, Y. Horsmans, O. Weiland, H.W. Reesink, L. Rodrigues, Y.B. Hu, T. Podsadecki, B. Bernstein, ABT-450, ritonavir, ombitasvir, and dasabuvir achieves 97% and 100% sustained virologic response with or without ribavirin in treatment-experienced patients with HCV genotype 1b infection, Gastroenterology. 147 (2014) 359–365.e1. doi:10.1053/j.gastro.2014.04.045.

[14] E. Lawitz, E. Gane, B. Pearlman, E. Tam, W. Ghesquiere, D. Guyader, L. Alric, J.-P. Bronowicki, L. Lester, W. Sievert, R. Ghalib, L. Balart, F. Sund, M. Lagging, F. Dutko, M. Shaughnessy, P. Hwang, A.Y.M. Howe, J. Wahl, M. Robertson, E. Barr, B. Haber, Efficacy and safety of 12 weeks versus 18 weeks of treatment with grazoprevir (MK-5172) and elbasvir (MK-8742) with or without ribavirin for hepatitis C virus genotype 1 infection in previously untreated patients with cirrhosis and patients with previous, Lancet. 385 (2014) 1075–1086. doi:10.1016/S0140-6736(14)61795-5.

[15] G.T. Everson, W.J. Towner, M.N. Davis, D.L. Wyles, R.G. Nahass, P.J. Thuluvath, K. Etzkorn, F. Hinestrosa, M. Tong, M. Rabinovitz, T. Morgan, R.T. Chung, T.T. Tran, Annals of internal medicine sofosbuvir with velpatasvir in treatment-naive noncirrhotic patients with genotype 1 to 6 hepatitis C virus infection, Ann. Intern. Med. 163 (2015) 818–826. doi:10.7326/M15-1000.

[16] J.O. Link, J.G. Taylor, L. Xu, M. Mitchell, H. Guo, H. Liu, D. Kato, T. Kirschberg, J. Sun, N. Squires, J. Parrish, T. Keller, Z. Yang, C. Yang, M. Matles, Y. Wang, K. Wang, G. Cheng, Y. Tian, E. Mogalian, E. Mondou, M. Cornpropst, J. Perry, M.C. Desai, Discovery of ledipasvir (GS-5885): A potent, once-daily oral NS5A inhibitor for the treatment of hepatitis C virus infection, J. Med. Chem. 57 (2014) 2033–2046.

[17] M.D. Schneider, C. Sarrazin, Antiviral therapy of hepatitis C in 2014: do we need resistance testing?, Antiviral Res. 105 (2014) 64–71. doi:10.1016/j.antiviral.2014.02.011.

[18] A.J. Thompson, S.A. Locarnini, M.R. Beard, Resistance to anti-HCV protease inhibitors., Curr. Opin. Virol. 1 (2011) 599–606. doi:10.1016/j.coviro.2011.10.001.

[19] J. Vermehren, C. Sarrazin, The role of resistance in HCV treatment., Best Pract. Res. Clin. Gastroenterol. 26 (2012) 487–503. doi:10.1016/j.bpg.2012.09.011.

[20] Guidelines for the screening, care, and treatment of persons with chronic hepatitis C infection; World Health Organization (WHO); Updated April 2016, (n.d.) http://www.who.int/hepatitis/publications/hepatiti.

[21] T. Asselah, N. Boyer, D. Saadoun, M. Martinot-Peignoux, P. Marcellin, Direct-acting antivirals for the treatment of hepatitis C virus infection: Optimizing current IFN-free treatment and future perspectives, Liver Int. 36 (2016) 47–57. doi:10.1111/liv.13027.

[22] R.T. Chung, G.L. Davis, D.M. Jensen, H. Masur, M.S. Saag, D.L. Thomas, A.I. Aronsohn, M.R. Charlton, J.J. Feld, R.J. Fontana, M.G. Ghany, E.W. Godofsky, C.S. Graham, A.Y. Kim, J.J. Kiser, S. Kottilil, K.M. Marks, P. Martin, K. Mitruka, T.R. Morgan, S. Naggie, D. Raymond, N.S. Reau, R.T. Schooley, K.E. Sherman, M.S. Sulkowski, H.E. Vargas, J.W. Ward, D.L. Wyles, Hepatitis C guidance: AASLD-IDSA recommendations for testing, managing, and treating adults infected with hepatitis C virus, Hepatology. 62 (2015) 932–954. doi:10.1002/hep.27950.

[23] V. Summa, S.W. Ludmerer, J. a McCauley, C. Fandozzi, C. Burlein, G. Claudio, P.J. Coleman, J.M. Dimuzio, M. Ferrara, M. Di Filippo, A.T. Gates, D.J. Graham, S. Harper, D.J. Hazuda, C. McHale, E. Monteagudo, V. Pucci, M. Rowley, M.T. Rudd, A. Soriano, M.W. Stahlhut, J.P. Vacca, D.B. Olsen, N.J. Liverton, S.S. Carroll, MK-5172, a selective inhibitor of hepatitis C virus NS3/4a protease with broad activity across genotypes and resistant variants., Antimicrob. Agents Chemother. 56 (2012) 4161–4167. doi:10.1128/AAC.00324-12.

[24] M.T. Rudd, C.J. McIntyre, J.J. Romano, J.W. Butcher, M.K. Holloway, K. Bush, K.T. Nguyen, K.F. Gilbert, T. a Lyle, N.J. Liverton, B.-L. Wan, V. Summa, S. Harper, M. Rowley, J.P. Vacca, S.S. Carroll, C. Burlein, J.M. DiMuzio, A. Gates, D.J. Graham, Q. Huang, S.W. Ludmerer, S. McClain, C. McHale, M. Stahlhut, C. Fandozzi, A. Taylor, N. Trainor, D.B. Olsen, J. a McCauley, Development of macrocyclic inhibitors of HCV NS3/4A protease with cyclic constrained P2-P4 linkers., Bioorg. Med. Chem. Lett. 22 (2012) 7207–13. doi:10.1016/j.bmcl.2012.09.061.

[25] M.T. Rudd, J.W. Butcher, K.T. Nguyen, C.J. McIntyre, J.J. Romano, K.F. Gilbert, K.J. Bush, N.J. Liverton, M.K. Holloway, S. Harper, M. Ferrara, M. DiFilippo, V. Summa, J. Swestock, J. Fritzen, S.S. Carroll, C. Burlein, J.M. DiMuzio, A. Gates, D.J. Graham, Q. Huang, S. McClain, C. McHale, M.W. Stahlhut, S. Black, R. Chase, A. Soriano, C.M. Fandozzi, A. Taylor, N. Trainor, D.B. Olsen, P.J. Coleman, S.W. Ludmerer, J.A. McCauley, P2-quinazolinones and bis-macrocycles as new templates for next-generation hepatitis C virus NS3/4a protease inhibitors: Discovery of MK-2748 and MK-6325, ChemMedChem. 10 (2015) 727–735. doi:10.1002/cmdc.201402558.

[26] P. Örtqvist, J. Gising, A.E. Ehrenberg, A. Vema, A. Borg, A. Karlén, M. Larhed, U.H. Danielson, A. Sandström, Discovery of achiral inhibitors of the hepatitis C virus NS3 protease based on 2(1H)-pyrazinones., Bioorg. Med. Chem. 18 (2010) 6512–6525. doi:10.1016/j.bmc.2010.06.101.

[27] J. Gising, A.K. Belfrage, H. Alogheli, A. Ehrenberg, E. Åkerblom, R. Svensson, P. Artursson, A. Karlén, U.H. Danielson, M. Larhed, A. Sandström, Achiral pyrazinone-based inhibitors of the hepatitis C virus NS3 protease and drug-resistant variants with elongated substituents directed toward the S2 pocket., J. Med. Chem. 57 (2014) 1790–1801. doi:10.1021/jm301887f.

[28] J. Gising, P. Örtqvist, A. Sandström, M. Larhed, A straightforward microwave method for rapid synthesis of N-1, C-6 functionalized 3,5-dichloro-2(1H)-pyrazinones., Org. Biomol. Chem. 7 (2009) 2809–2815. doi:10.1039/b905501k.

[29] A.K. Belfrage, J. Gising, F. Svensson, E. Åkerblom, C. Sköld, A. Sandström, Efficient and selective palladium-catalysed C-3 urea couplings to 3,5-dichloro-2(1H)-pyrazinones, European J. Org. Chem. 2 (2015) 978–986. doi:10.1002/ejoc.201403405.

[30] K.X. Chen, L. Nair, B. Vibulbhan, W. Yang, A. Arasappan, S.L. Bogen, S. Venkatraman, F. Bennett, W. Pan, M.L. Blackman, A.I. Padilla, A. Prongay, K.-C. Cheng, X. Tong, N.-Y. Shih, F.G. Njoroge, Second-generation highly potent and selective inhibitors of the hepatitis C virus NS3 serine protease., J. Med. Chem. 52 (2009) 1370–1379. doi:10.1021/jm801238q.

[31] S. Venkatraman, F.G. Njoroge, W. Wu, V. Girijavallabhan, B. McKittrick, J. Su, F. Velazquez, P.A. Pinto, WO 2009/058856 A1, 2009.

[32] C.A. Busacca, D. Grossbach, E. Spinelli, A convenient synthesis of (1S)-tert-butyl-1, 2-ethylenediamine, Tetrahedron:Asymmetry. 11 (2000) 1907–1910.

[33] S. Venkatraman, M. Blackman, W. Wu, L. Nair, A. Arasappan, A. Padilla, S. Bogen, F. Bennett, K. Chen, J. Pichardo, X. Tong, A. Prongay, K.-C. Cheng, V. Girijavallabhan, F. George Njoroge, Discovery of novel P3 sulfonamide-capped inhibitors of HCV NS3 protease. Inhibitors with improved cellular potencies., Bioorg. Med. Chem. 17 (2009) 4486–95. doi:10.1016/j.bmc.2009.05.012.

[34] S. Perveen, S.M. Abdul Hai, R. a. Khan, K.M. Khan, N. Afza, T.B. Sarfaraz, Expeditious method for synthesis of symmetrical 1,3-disubstituted ureas and thioureas, Synth. Commun. 35 (2005) 1663–1674. doi:10.1081/SCC-200061656.

[35] A.K. Belfrage, E. Abdurakhmanov, E. Åkerblom, P. Brandt, A. Oshalim, J. Gising, A. Skogh, J. Neyts, U.H. Danielson, A. Sandström, Discovery of pyrazinone based compounds that potently inhibit the drug-resistant enzyme variant R155K of the hepatitis C virus NS3 protease, Bioorg. Med. Chem. 24 (2016) 2603–2620. doi:10.1016/j.bmc.2016.03.066.

[36] A. Poliakov, I. Hubatsch, C.F. Shuman, G. Stenberg, U.H. Danielson, Expression and purification of recombinant full-length NS3 protease-helicase from a new variant of Hepatitis C virus., Protein Expr. Purif. 25 (2002) 363–371. http://www.ncbi.nlm.nih.gov/pubmed/12182815.

[37] G. Dahl, A. Sandström, E. Åkerblom, U.H. Danielson, Resistance profiling of hepatitis C virus protease inhibitors using full-length NS3, Antivir. Ther. 12 (2007) 733–740.

[38] V. Lohmann, F. Körner, J.-O. Koch, U. Herian, L. Theilmann, R. Bartenschlager, Replication of Subgenomic Hepatitis C Virus RNAs in a Hepatoma Cell Line, Science (80-.). 285 (1999) 110–114.

[39] P. Örtqvist, S.D. Peterson, E. Åkerblom, T. Gossas, Y.A. Sabnis, R. Fransson, G. Lindeberg, U. Helena Danielson, A. Karlén, A. Sandström, Phenylglycine as a novel P2 scaffold in hepatitis C virus NS3 protease inhibitors., Bioorg. Med. Chem. 15 (2007) 1448–74.

[40] M. Gallo, M.J. Bottomley, M. Pennestri, T. Eliseo, M. Paci, U. Koch, R. Bazzo, V. Summa, A. Carfi, D.O. Cicero, Structural characterization of the Hepatitis C Virus NS3 protease from genotype 3a: the basis of the genotype 1b vs. 3a inhibitor potency shift., Virology. 405 (2010) 424–38. doi:10.1016/j.virol.2010.05.035.

[41] M. Kramer, D. Halleran, M. Rahman, M. Iqbal, M.I. Anwar, S. Sabet, E. Ackad, M. Yousef, Comparative molecular dynamics simulation of Hepatitis C Virus NS3/4A protease (Genotypes 1b, 3a and 4b) predicts conformational instability of the catalytic triad in drug resistant strains, PLoS One. 9 (2014) e104425. doi:10.1371/journal.pone.0104425.

[42] N. Schiering, A.D. Arcy, F. Villard, O. Simi, M. Kamke, G. Monnet, U. Hassiepen, A macrocyclic HCV NS3 / 4A protease inhibitor interacts with protease and helicase residues in the complex with its full-length target, PNAS. 108 (2011) 21052–21056. doi:10.1073/pnas.1110534108/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1110534108.

[43] N. Yao, P. Reichert, S.S. Taremi, W.W. Prosise, P.C. Weber, Molecular views of viral polyprotein processing revealed by the crystal structure of the hepatitis C virus bifunctional protease – helicase, Structure. 7 (1999) 1353–1363.

[44] K.P. Romano, A. Ali, W.E. Royer, C.A. Schiffer, Drug resistance against HCV NS3/4A inhibitors is defined by the balance of substrate recognition versus inhibitor binding., Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 20986–20991. doi:10.1073/pnas.1006370107.

[45] K.P. Romano, A. Ali, C. Aydin, D. Soumana, A. Ozen, L.M. Deveau, C. Silver, H. Cao, A. Newton, C.J. Petropoulos, W. Huang, C.A. Schiffer, The molecular basis of drug resistance against hepatitis C virus NS3/4A protease inhibitors., PLoS Pathog. 8 (2012) 1–15. doi:10.1371/journal.ppat.1002832.

[46] R. Rönn, Y.A. Sabnis, T. Gossas, E. Åkerblom, U.H. Danielson, A. Hallberg, A. Sandström, Exploration of acyl sulfonamides as carboxylic acid replacements in protease inhibitors of the hepatitis C virus full-length NS3, Bioorg. Med. Chem. 14 (2006) 544–559.

[47] A. Johansson, A. Poliakov, E. Åkerblom, G. Lindeberg, S. Winiwarter, B. Samuelsson, H.U. Danielson, A. Hallberg, Tetrapeptides as Potent Protease Inhibitors of Hepatitis C Virus, Bioorg. Med. Chem. 10 (2002) 3915–3922.

[48] E. Harder, W. Damm, J. Maple, C. Wu, M. Reboul, J.Y. Xiang, L. Wang, D. Lupyan, M.K. Dahlgren, J.L. Knight, J.W. Kaus, D.S. Cerutti, G. Krilov, W.L. Jorgensen, R. Abel, R.A. Friesner, OPLS3: A Force Field Providing Broad Coverage of Drug-like Small Molecules and Proteins., J. Chem. Theory Comput. 12 (2016) 281–296. doi:10.1021/acs.jctc.5b00864.

[49] W.C. Still, A. Tempczyk, R.C. Hawley, T. Hendrickson, Semianalytical treatment of solvation for molecular mechanics and dynamics, J. Am. Chem. Soc. 112 (1990) 6127–6129. doi:10.1021/ja00172a038.

- R^3-elongated 2(1*H*)-pyrazinone based HCV NS3 protease inhibitors were synthesized.
- Nanomolar inhibitory potencies against genotype 1a, wt and R155K, and genotype 3a.
- Compound **38**: K_i (1a) = 30 nM, K_i (R155K) = 2 nM and K_i (3a) = 5 nM).