Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 1005–1008

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

# Azapeptides as Inhibitors of the Hepatitis C Virus NS3 Serine Protease

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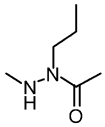
Received 4 January 2002; accepted 1 February 2002

**Abstract**—Truncation and substitution SAR studies of azapeptide-based inhibitors of the Hepatitis C virus (HCV) NS3 serine protease have been performed. These azapeptides were designed from the HCV polyprotein's NS5A-NS5B *trans* cleavage junction and contained an azaamino acid residue at the P1 position. These azapeptides exhibited predominantly non-acylating, competitive inhibition, contrary to classical azapeptides. 

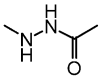
Hepatitis C virus (HCV) infects ~1% of the global population and causes chronic hepatitis, liver cirrhosis, hepatocellular carcinoma, and liver failure.[1–3] Current α-interferon monotherapy and α-interferon–ribavirin combination therapy are only effective in ~25 and ~50% of the cases, respectively.[4,5] For this reason, new and more effective anti-HCV therapies are urgently needed.

HCV is a small enveloped virus containing a positive-stranded linear RNA genome that encodes a polyprotein of ~3000 amino acids.[6] The processing of the HCV polyprotein into both structural and nonstructural (NS) proteins is mediated by host signal peptidases and two viral proteases, the NS2 metalloproteinase and NS3 serine protease. NS2 metalloproteinase auto-catalytically cleaves the NS2–NS3 junction. The NS3 serine protease[7] is contained within the N-terminal third of the multifunctional NS3 protein. The protease performs *cis* cleavage at the NS3–NS4A junction, and *trans* cleavage at the NS4A–NS4B, NS4B–NS5A, and NS5A–NS5B junctions. Structural determinations confirmed that the NS3 protease is a chymotrypsin-like serine protease.[8–10] The structure and mechanism-based inhibitor design for NS3 serine protease presents an attractive new route for anti-HCV therapy.

This report describes the use of azapeptides to inhibit the HCV NS3 serine protease.[11] An azapeptide[12] contains at least one azaamino acid residue in which the α-carbon of an amino acid is replaced by a nitrogen. Azapeptides have been used as inhibitors for both serine and cysteine proteases.[13–17] We initially prepared a short series of azapeptides[18] (Table 1) containing aza-norvalyl (AzaNva) or azaglycyl (AzaGly) at the P1 position and Pro at P2 to minimize aggregation, with the remaining sequence identical with the NS5A–NS5B peptide sequence found in the HCV-1a subtype. These azapeptides exhibited competitive inhibition kinetics in a continuous spectrophotometric assay.[19] The inhibition constants ($K_i$) for compounds **1**–**3** were comparable to the Michaelis–Menten constant of the NS5A–NS5B derived peptide substrate, DTEDVVCCSM-SYTWTGK-OH ($K_m = 20 \pm 2$ μM).[20] Azaglycyl substitution at P1 (**6**) abrogated the binding affinity, highlighting the critical importance of the P1 residue in protease binding.[20,21] C-Terminal truncations from P8′ to P3′ weakened the potency by 10- to 20-fold (**4** and **5**).



AzaNva



AzaGly

Another set of azapeptides containing nonpeptide P1′ groups [18] was prepared to explore the P1′ SAR (Table 2). The binding affinities of these azapeptides varied by >500-fold depending on the nature of the P1′ group and the chemical linkage. Replacement of a scissile amide group by a thioester (**11** and **12**), ketone (**15**–**27** and **31**–**33**), α-ketoester (**34**), or sulfonyl group (**35**)

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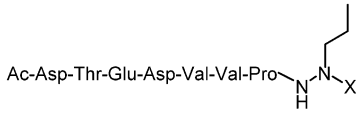
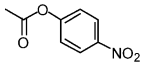
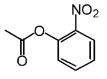
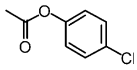
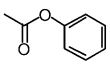
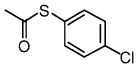
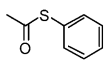
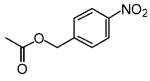
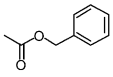
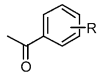
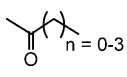
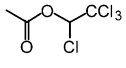
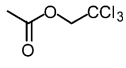
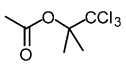
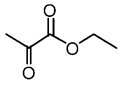
0960-894X/02/$ - see front matter 
PII: S0960-894X(02)00102-6

**Table 1.** Substrate-based azapeptides

| Peptide | Structure | $K_i$ (μM) |
|---|---|---|
| | P8 P7 P6 P5 P4 P3 P2 P1 P1′ P2′ P3′ P4′ P5′ P6′ P7′ P8′ | |
| **1** | Asp-Thr-Glu-Asp-Val-Val-Pro-(**AzaNva**)-Ser-Met-Ser-Tyr-Thr-Trp-Thr-Gly-Lys-OH | 20±5 |
| **2** | Ac-Asp-Thr-Glu-Asp-Val-Val-Pro-(**AzaNva**)-Ser-Met-Ser-Tyr-Thr-Trp-Thr-Gly-Lys-OH | 10±2 |
| **3** | Ac-Glu-Asp-Val-Val-Pro-(**AzaNva**)-Ser-Met-Ser-Tyr-Thr-Trp-Thr-Gly-Lys-OH | 20±5 |
| **4** | Ac-Glu-Asp-Val-Val-Pro-(**AzaNva**)-Ser-Met-Ser-OH | 200±50 |
| **5** | Ac-Asp-Thr-Glu-Asp-Val-Val-Pro-(**AzaNva**)-Ser-Met-Ser-OH | 200±50 |
| **6** | Ac-Asp-Thr-Glu-Asp-Val-Val-Pro-(**AzaGly**)-Ser-Met-Ser-Tyr-Thr-Trp-Thr-Gly-Lys-OH | >200 |

**Table 2.** P1′ SAR of P1 AzaNva-containing peptides

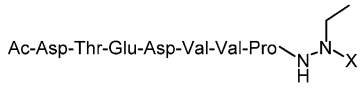


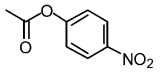
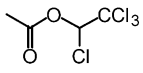

| Peptide | X | $K_i$ (μM) | Peptide | X | $K_i$ (μM) |
|---|---|---|---|---|---|
| **7** |  | 2.0±0.2 | **8** |  | 9±1 |
| **9** |  | 16±2 | **10** |  | 8±1 |
| **11** |  | 100±20 | **12** |  | 160±20 |
| **13** |  | 45±5 | **14** |  | 80±10 |
| **15–23**[a] |  | >100 | **24–27** |  | >100 |
| **28** |  | 0.2±0.1 | **29** |  | 14±2 |
| **30** |  | >100 | **31** |  | >100 |
| **32** |  | >100 | **33** |  | >100 |
| **34** |  | >100 | **35** |  | >100 |

[a]**15–23**: R = 4-trifluoromethyl (**15**), 3,5-bis-trifluoromethyl (**16**), pentafluoro (**17**), 4-cyano (**18**), 3,5-dinitro (**19**), 4-nitro (**20**), 4-fluorosulfonyl (**21**), 4-(4′-nitrophenyl)azo (**22**), 4-phenyl (**23**).

yielded weak or inactive azapeptides, whereas incorporation of an ester group afforded more active molecules (**7**–**10**, **13**, **14**, **28**, **29**, but not **30**). Within the ester series, we observed that compounds with a better leaving group were the more potent inhibitors such that **28** and **7** were the two most potent. We have found that, regardless of the differences in the chemical reactivities of the P1′ groups, the azapeptides consistently exhibited competitive inhibition kinetics in the continuous assay.

**Table 3.** P1 AzaAbu-containing peptides




| Peptide | X | $K_i$ (μM) | Peptide | X | $K_i$ (μM) |
|---|---|---|---|---|---|
| **36** |  | >100 | **37** |  | 0.9±0.1 |

No obvious curvatures were observed by progress curve analysis, suggesting that during the assay time frame (0–30 min) there was no significant time-dependent inactivation or slow binding resulting from the potential acylation of the protease by some of the more active azapeptide esters (see more discussion later). Thus, the measured inhibition constants appeared to reflect the equilibrium binding between the protease and azapeptide esters.

The *n*-propyl side chain of the P1 AzaNva residue was chosen as a non-reactive surrogate for the native P1 Cys side chain based on our observation that peptide product inhibitors (unpublished data) containing P1Nva (*n*-propyl side chain) were more potent than those containing either Abu (ethyl side chain) or Nle (*n*-butyl side chain). When P1 AzaAbu was used in place of AzaNva (Table 3), there was a significant reduction in potency (cf., **36** with **7**, and **37** with **28**). In a similar study, the P1 side chains were found to exert quite different effects on selectivity and potency.[11]

To directly measure the turnover kinetics of azapeptides and to evaluate the kinetic effect of the azaaminio acid, we designed a non-azapeptide 4-nitrophenyl ester (**38**) and several N-terminally truncated azapeptidyl 4-nitrophenyl esters (Table 4). The proteolytic turnover kinetics were measured by following the chromogenic 4-nitrophenol formation spectroscopically at 400 nm.[22] The background autohydrolysis of these azapeptides at pH 6.5 was <1% of the proteolytic turnover rate. The azaamino acid reduced the proteolytic turnover rate by ~200-fold (compare the $k_{cat}$ values for **38** and **7**) while the ground state binding was not affected (cf., the $K_m$ values for **38** and **7**). Progressive N-terminal truncations from P8 to P5 (**7**, **39**–**41**) had little effect on the inhibi-

**Table 4.** Turnover kinetics and inhibition constants for peptide-4-nitrophenyl (4Np) esters

| Peptide | Structure | $k_{cat}$ ($min^{-1}$) | $K_m$ (μM) | $K_i$ (μM) |
|---|---|---|---|---|
| **38** | Ac-DTEDVVP(**Nva**)-*O*-4Np | 100±10 | 4±1 | Not determined |
| **7** | Ac-DTEDVVP(**AzaNva**)-*O*-4Np | 0.5±0.1 | 5±1 | 3±1 |
| **39** | Ac-TEDVVP(**AzaNva**)-*O*-4Np | 0.5±0.1 | 8±2 | 5±1 |
| **40** | Ac-EDVVP(**AzaNva**)-*O*-4Np | 0.5±0.1 | 4±1 | 6±1 |
| **41** | Ac-DVVP(**AzaNva**)-*O*-4Np | 0.5±0.1 | 9±2 | 7±2 |
| **42** | Ac-VVP(**AzaNva**)-*O*-4Np | 0.4±0.1 | 100±20 | 100±20 |

tion potency and turnover kinetics, whereas further truncation to P4 (**42**) decreased the binding by ~10-fold. The $K_m$ values determined in the turnover kinetics agreed well with the $K_i$ values determined independently from inhibition kinetics.

Finally, we investigated the nature of the inhibition process by these azapeptides, which displayed competitive inhibition kinetics. To probe whether the azapeptides formed an acylation complex with the NS3 serine protease, we used direct infusion electrospray ionization mass spectrometry (ESI MS) to detect the changes in MW of the NS3 serine protease after it was pre-incubated (at 60 μM on ice for 0.5–48 h) with compound **2**, **5**, **7**, **8**, or **14** (140 μM) and was rapidly desalted by size-exclusion chromatography. We observed mass increases which corresponded exactly to acylation by **7**, **8**, or **14** and a noncovalent complex with **2** or **5**, in addition to MW of the free protease. When the pre-incubated and desalted NS3 serine protease was denatured by adding an equal volume of pure acetonitrile (accompanied by loss of the structural $Zn^{2+}$), azapeptide **2** or **5** dissociated completely from the noncovalent complex whereas the acyl portion of **7**, **8**, or **14** remained bound to the protease, as expected. Detection of the acylated protease intermediate under the above conditions after two days suggested that deacylation was quite slow and that the pheyl esters in compounds **7**, **8**, and **14** must have partially restored the electrophilicity of the P1 azaamino acid's carbonyl to allow acylation to occur. However, we estimated that <10% of the protease was trapped in an inactive, acylated state since <10% time-dependent inactivation was observed with the protease when assayed after pre-incubation with selected azapeptides (**7**, **28**, and **29**) and dilution into assay buffer. HPLC analysis revealed insignificant accumulation of the proteolytic products (peptide hydrazides) from compounds **7**, **28**, and **29** during a standard 30-min assay that used 1–2 nM protease. Taken together, as a result of the P1 azaamino acid-induced decrease of both acylation and deacylation rates, the proteolytic turnover rates (Table 4, and ~4/h for **28** and **29**) were reduced to the extent that these azapeptides were predominantly non-acylating, competitive inhibitors, contrary to the slow-binding behavior exhibited by other azapeptides.[13–17]

## Acknowledgements

We thank Dr. Yan-hui Liu for performing the ESI MS analysis, Drs. Bruce A. Malcolm and Tin-Yau Chan for critical reading for the manuscript, and Drs. Hung Le and Patricia C. Weber for support.

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17. Baggio, R.; Shi, Y.-Q.; Wu, Y.-q.; Abeles, R. H. *Biochem.* **1996**, *35*, 3351.
18. **Azapeptide synthesis**. Azapeptides were prepared using Fmoc-protected amino acid carbazate dipeptide synthons, $N^1$-(Fmoc-Prolyl)-$N^2$-monoalkyl-hydrazine (following Quibell,

M.; Trunell, W. G.; Johnson, T. *J. Chem. Soc., Perkin Trans. 1* **1993**, 2483). Briefly, Fmoc-Pro-pentafluorophenyl ester was reacted quantitatively with hydrazine monohydrate (1 mol equiv) in dichloromethane to form Fmoc-Pro-NH-$NH_2$ followed by a two-step reductive alkylation (hydrazone formation with aldehyde in dry THF overnight and then reduction by sodium cyanoborohydride in ethanol containing 1% acetic acid). The monoalkylated dipeptide synthon species was purified by FLASH chromatography and confirmed by mass and NMR analysis. The azapeptides in Table 1 were assembled on solid phase and the dipeptide synthon was coupled via either the *C*-activation approach using triphosgene (see Andre, F.; Marraud, M.; Tsouloufis, T.; Tzaros, S. J.; Boussard, G. *J. Peptide Sci.* **1997**, *3*, 429) or the *N*-activation approach using 4-nitrophenyl chloroformate (see Gante, J. *J. Chem. Ber.* **1965**, *98*, 3340). To prepare the azapeptides containing nonpeptide P1′ groups (Tables 2–4), the dipeptide synthon was first anchored to 2-chlorotrityl chloride resin (Barlos, K.; Chatzi, O.; Gatos, D.; Stavropoulos, G. *Int. J. Peptide Protein Res.* **1991**, *37*, 513) and the rest of the peptide sequence assembled on the solid support. The protected peptide segment was cleaved off the resin using 2% trifluoroacetic acid in dichloromethane for 10 min followed by acid extraction with sodium carbonate in brine and dried over sodium sulfate. Various commercial chloroformating agents (2 mol equiv) were reacted with the protected peptide hydrazides in dichloromethane in the presence of *N*-methylmorpholine (4 mol equiv). The reaction was quenched by 4% hydrochloride (aq) and the dichloromethane phase was evaporated before final deprotection with 95% trifluoroacetic acid. The azapeptides were purified by HPLC and confirmed by mass analysis.

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22. **Determinations of $k_{cat}$, $K_m$, and $K_i$ values.** $K_i$ values were determined in a continuous assay as described[19] using the NS4A-tethered single chain NS3 serine protease (Taremi, S. S.; Beyer, B.; Maher, M.; Yao, N.; Prosise, W.; Weber. P. C.; Malcolm, B. A. *Protein Sci.* **1998**, *7*, 2143). The autohydrolytic cyclization product (substituted oxadiazolones)[16] and the proteolytic product (peptide hydrazides) were not inhibitory against the HCV NS3 serine protease at concentrations up to 100 μM. For the determination of $k_{cat}$ and $K_m$ values in Table 4, the initial velocities of the chromogenic product (4-nitrophenol) formation, which was continuously monitored at 400 nm, were measured at different concentrations ($\sim$0.5 $K_m$ to 8 $K_m$) of the starting azapeptide. The initial velocities were then fitted into the steady state Michaelis–Mention kinetic equation, $v_0 = V_{max}[S]/(K_m + [S])$, where $v_0$ is the initial velocity, $V_{max}$ is the maximal velocity, $K_m$ is the Michaelis–Menten constant, [S] is the substrate concentration. The $k_{cat}$ values were then calculated from $V_{max}/[E]_T$, where $[E]_T$ is the total concentration of the protease which was assumed to be fully active.