Non-peptide inhibitors of HCV serine proteinase

Nobuko Kakiuchi[a,*], Yasumasa Komoda[a], Keiko Komoda[a], Norisue Takeshita[a], Satoshi Okada[a], Tadato Tani[a], Kunitada Shimotohno[b]

[a] *HQL Research Laboratories, Sumitomo Metal Industries, Kyoto 619-02, Japan*
[b] *Institute for Virus Research, Kyoto University, Kyoto 606-01, Japan*

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Abstract We screened a chemical library of 2000 compounds for inhibitors of hepatitis C virus (HCV) serine proteinase using an in vitro screening method measuring the hydrolysis of the peptide substrate. Three compounds were found to be the most potent inhibitors ($IC_{50} < 10^{-5}$ M). Two of them had a similar structure, that of halogenated benzanilide, and were not inhibitory for common serine proteinases. They inhibited the enzyme non-competitively with the substrate. Together with the result of the analogous compounds in the chemical library, the presumed structural requirements of the inhibition are pointed out.

Key words: Hepatitis C virus; Proteinase inhibitor; Non-peptide compound

1. Introduction

Hepatitis C virus (HCV) is an etiological agent of non-A non-B hepatitis. The amino acid sequence deduced from cDNA cloned in recent years has revealed that the virus is closely related to the Flaviviridae [1–4]. The polyprotein generated from the HCV genome is predicted to be organized as 5′-C-E1-E2-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3′. Two HCV viral encoding proteinases have been identified: Cpro-1 and Cpro-2 [5]. The latter is the trypsin-like serine proteinase and is located at the N-terminal one-third of NS3, whereas the former is thought to be a metalloproteinase which is responsible for the cleavage at the NS2/NS3 junction. Since viral proteinases are indispensable for virus propagation, they are good targets for antiviral agents. For screening of viral proteinase inhibitors, a rapid and sensitive assay of enzyme activity is required. In a previous article, we reported an enzyme-linked immunosorbent assay (ELISA) using a fusion protein of the HCV NS3 serine proteinase with maltose binding protein (MBP-NS3) produced in *Escherichia coli*, and a streptavidin-coated microtiter plate which can trap the C-terminal biotinylated product as well as substrate [6]. The free amino moieties generated from the C-terminal half of the product were quantified. The method has a high throughput capacity and can be automated, and is thus suited for the screening of a large number of samples. We report here the result of the screening of 2000 compounds.

*Corresponding author. Present address: Institute for Virus Research, Kyoto University, 53 Showgoin Kawaharamachi, Sakyo-ku, Kyoto 606-01, Japan. Fax: (81) (75) 751-3998.
E-mail: nkakiuch@virus.kyoto-u.ac.jp

Abbreviations: Ac, acetyl; Boc, *t*-butyloxycarbonyl; Bz, benzoyl; DMSO, dimethyl sulfoxide; Dns, dansyl; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; HCV, hepatitis C virus; HPLC, high-performance liquid chromatography; MBP, maltose binding protein; MCA, 4-methyl-coumaryl-7-amide; NS, non-structural protein; pNA, *p*-nitroanilide; Pyr, L-pyroglutamyl; SDS, sodium dodecylsulfate; Suc, succinyl; Z, carbobenzoxy

2. Materials and methods

The synthetic compounds included in the HQL Chemical Library were purchased from Summit Pharmaceuticals International Co. (Tokyo, Japan).

2.1. Production and preparation of the fused form of NS3 serine proteinase (MBP-NS345Ps)

The expression vector containing the amino acid sequence of HCV polypeptide from 985 to 2052, which covered NS3, NS4A, NS4B and the N-terminus of NS5A, in a plasmid pMAL-c (New England Biolabs, Beverly, MA) was constructed by the replacement of the pMANS2d3X [7] *Sac*II-*Sal*I fragment with that of the entire HCV non-structural region of the genome followed by *Pst*I deletion. The expression of the enzyme in *E. coli* was performed according to the method reported previously [8]. The enzyme was purified by affinity purification carried out as reported [8], and then chromatographed on a Hi-Trap Sepharose column followed by gel filtration with a Sephacryl S-300 column. The purity of the enzyme was found to be more than 70% by SDS-polyacrylamide gel electrophoresis analysis quantified with a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA).

2.2. HCV serine proteinase assay

The peptide substrates Ac-Gly-Glu-Ala-Gly-Asp-Asp-Ile-Val-Pro-Cys-Ser-Met-Ser-Tyr-Thr-Trp-Thr-Lys-Biotin (AcP17Bio) and Dns-Gly-Glu-Ala-Gly-Asp-Asp-Ile-Val-Pro-Cys-Ser-Met-Ser-Tyr-Thr-Trp-Thr-OH (Pep5) were obtained from the Peptide Institute Co. (Osaka, Japan) and dissolved in DMSO. The standard enzyme reaction for ELISA was performed as follows: MBP-NS345Ps 80 μg/ml in a buffer consisting of 50 mM Tris-HCl (pH 7.8), 5 mM $CaCl_2$, 10 mM DTT and 30 mM NaCl was preincubated with DMSO as control or with inhibitors dissolved in DMSO for 15 min at 37°C. The reaction was started by addition of the AcP17Bio solution at a final concentration of 86 μM (5% final DMSO concentration). The reaction was prolonged for 30 min and then quenched by addition of three volumes of DMSO. The reaction for the HPLC analysis was done using Pep5 at a 2% DMSO concentration for 10 min, and stopped by addition of 5 N NaOH.

2.3. Proteinase assay

The proteinases were purchased as follows: chymotrypsin (bovine pancreas, Wako Pure Chemicals, Osaka), factor Xa (bovine plasma, Takara Shuzo, Otsu, Japan), kallikrein (human urine, Funakoshi, Tokyo), plasmin (human plasma, Funakoshi), α-thrombin (bovine plasma, Sigma Chemicals, St. Louis, MO), and trypsin (porcine pancreas, Wako). The enzyme assay was performed as follows. Chymotrypsin 1 unit/ml was preincubated with a sample at 37°C for 10 min in a reaction mixture consisting of 50 mM Tris-HCl at pH 8.0 and 2 mM $CaCl_2$ and then reacted with Suc-Ala-Ala-Pro-Phe-MCA 2 μmol/ml for 10 min. Factor Xa 1 μg/ml was preincubated at 37°C for 20 min and then reacted with Z-Pyr-Gly-Arg-MCA. Kallikrein 1 unit/ml was preincubated at 37°C for 10 min and then reacted with Z-Phe-Arg-MCA 100 nmol/ml for 20 min. Plasmin 12 pK_{cat}/ml was preincubated at 37°C for 15 min and then reacted with Boc-Glu-

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Lys-Lys-MCA 200 nmol/ml for 20 min. Thrombin 10 units/ml was preincubated at 25°C for 15 min and treated with Boc-Val-Pro-Arg-MCA 400 nmol/ml for 20 min. Trypsin 5 units/ml was preincubated with 25°C for 15 min and then treated with Bz-Phe-Val-Arg-pNA for 20 min. The enzyme reaction was quenched by addition of 1% TCA and fluorescence (excitation 380 nm, emission 460 nm) or absorbance (405 nm) was then measured.

2.4. ELISA procedure

The ELISA procedure except for the coloring reaction was automatically performed according to the method previously reported [6] using a Biomek 1000 Automated Laboratory Workstation (Beckman Instruments Inc., Fullerton, CA).

3. Results and discussion

The enzyme used in this study was expressed as a fusion protein of MBP and HCV non-structural protein domain consisting of the amino acid sequence from residue 985 to 2052 in *E. coli*. The expressed protein was expected to be auto-cleaved at the NS3-NS4A, NS4A-NS4B and NS4B-NS5A junctions to produce MBP-NS3, NS4A, NS4B and the N-terminal part of NS5A. The major protein isolated by affinity chromatography for MBP had a molecular weight of about 120 kDa as judged from native polyacrylamide gel electrophoresis. This protein, designated MBP-NS345Ps, was found to be a complex of the MBP-NS3 (110 kDa) and NS4A (6 kDa) proteins, since it reacted with the anti-MBP, -NS3 and -NS4A antibodies. Furthermore, a protein signal of about 6 kDa which reacted with the anti-NS4A antibody was found in SDS-polyacrylamide gel electrophoresis analysis. This result confirmed that auto-cleavage at NS3-4A and NS4A-4B had occurred and that the free NS4A was bound to the NS3. In the case of the enzyme preparation we reported previously, MBP-NS3-4A, which contained the HCV amino acids 1027–1711, NS4A was covalently linked to NS3 [9]. The catalytic rate (K_{cat}) of the cleavage of Pep5 with MBP-NS345Ps at 37°C was 15.0. The value was 12 and three times as high as those of MBP-NS3 alone and the mixture of the MBP-NS3 and a fragment of the NS4A, P41 (MBP-NS3/P41) [10], respectively, and was almost comparable with the mixture of the N-terminal portion of NS3 (ΔNS3) and P41 (ΔNS3/P41) [11]. Unlike ΔNS3, MBP-NS345Ps was obtained as a soluble protein at neutral pH. Furthermore, the enzyme was more thermostable than MBP-NS3 or MBP-NS3/P41, and the optimum temperature of the cleavage of Pep5 was found to be 50°C, whereas those of the MBP-NS3 and MBP-NS3/P41 were 25°C and 37°C, respectively [10]. This evidence suggests that MBP-NS3 formed a tighter complex with NS4A than with the synthetic NS4A fragment and the complex had higher proteolytic activity. By using this enzyme, we were able to reduce the reaction time to half and the amount of the substrate to one quarter of the previous condition [6].

Two thousand synthetic compounds were randomly selected and screened. The first screening was performed at 10^{-4} M in a duplicated enzyme reaction. The reaction mixtures were assayed in duplicate by the automated ELISA. It is expected that 250 samples could be screened within a week with this method. The samples which reduced the cleavage to less than 30% of the control were selected and re-screened by HPLC analysis. After the two-step selection, three compounds, designated compounds **1**, **2** and **3**, were selected as the most effective inhibitors. Table 1 shows the results of the HPLC analysis of these compounds at concentrations of 10^{-4}, 10^{-5}, and 10^{-6} M as well as the IC_{50} values. As shown in Table 2, their effect on some serine proteases, including chymotrypsin, factor Xa, kallikrein, plasmin, α-thrombin and trypsin, were examined at a concentration of 10^{-5} M, where they reduced the HCV serine protease activity by more than 50%. A 50% or greater reduction of the activity of factor Xa and trypsin was produced by compound **1**. The other two compounds did not inhibit these proteinases effectively. Compounds **2** and **3**, which seemed to be specific inhibitors for HCV serine proteinase, were found to have a similarity in chemical structure, that of benzanilide, as shown in Fig. 1. The structure consisted of benzoic acid which was substituted with halogen and a hydroxy or amino group at the *meta* and *ortho* positions, respectively, and aniline whose *meta* position was chlorinated.

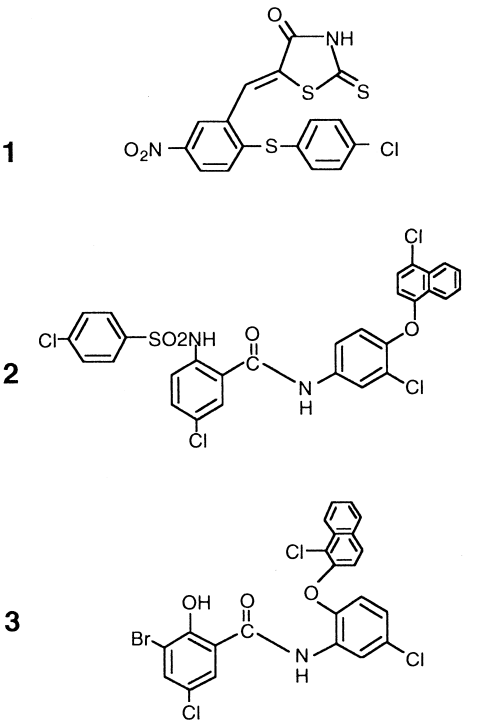

Fig. 1. The structure of compounds selected from the chemical library.

Table 1
Effect of the compounds selected from the chemical library on HCV serine proteinase

Compound	(%) Cleavage			IC_{50}
	10^{-4} M	10^{-5} M	10^{-6} M	$\times 10^{-5}$ (M)
1	1.9 ± 0.4[a]	18.6 ± 1.0	81.4 ± 6.4	0.32
2	15.5 ± 6.8	41.9 ± 10.2	85.2 ± 1.1	0.65
3	16.4 ± 7.5	35.6 ± 10.6	106.4 ± 11.9	0.62

[a]The values of production of the cleavage product relative to the control are expressed as means ± standard deviations of triplicate assays.

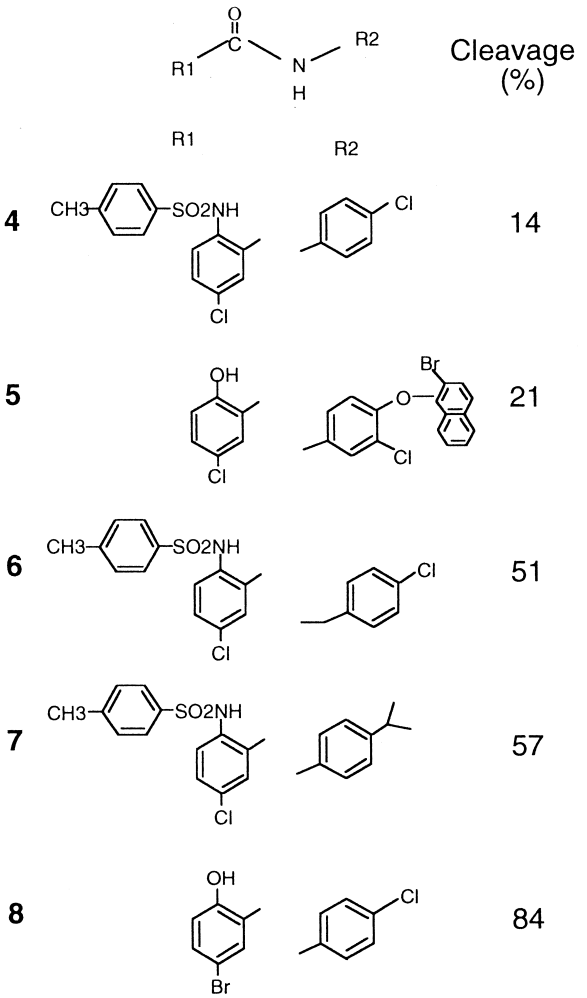

Fig. 2. The structure of the reference compounds in the chemical library and the (%) control of the cleavage activity of MBP-NS345Ps in the presence of these compounds at a concentration of 10^{-4} M.

To examine the effect of substituents on the inhibitory activity, compounds with analogous structures in the chemical library were compared as a reference. Fig. 2 shows the structure of the reference compounds and the result of the screening at a concentration of 10^{-4} M. Compound **5** had the same structural features of **2** and **3** as pointed out above, and so had **4** though the position of chlorine in the aniline moiety was *para* instead of *meta*, and they were almost as effective as **2** and **3**. Compounds **4**, **6** and **7** had the same R1 substituent, and different substituents in the aniline ring were compared. The insertion of methylene at the amino moiety (compound **6**) or the replacement of chlorine at the *para* position with isopropyl (compound **7**) reduced the inhibitory activity. It seems that the amino moiety conjugated with a phenyl ring and chlorine substitution favored the inhibition. Comparing compounds **3**, **5** and **8**, compounds which had the halogenated naphthoxy moiety in the aniline ring (compounds **3** and **5**) showed high inhibitory activity, and the replacement of this moiety with chlorine (compound **8**) resulted in a large reduction of the activity. Similarly, by the replacement of the phenyl sulfamide moiety in benzoyl ring of compound **4** with a smaller substituent, a hydroxyl group (compound **8**), the inhibitory activity was also reduced. From these results, common structural features of compounds **2** and **3**, as well as a large substitution in either the aniline or the benzoyl ring seem to be required for the inhibitory activity. The style of the inhibition of compound **3** was studied by Dixon plot as shown in Fig. 3. Compound **3** was found to be a non-competitive inhibitor as was compound **2** and their inhibition constant (K_i) values were estimated to be 3.6 and 2.4 μM, respectively. The results suggest that these compounds do not bind to the substrate binding pocket.

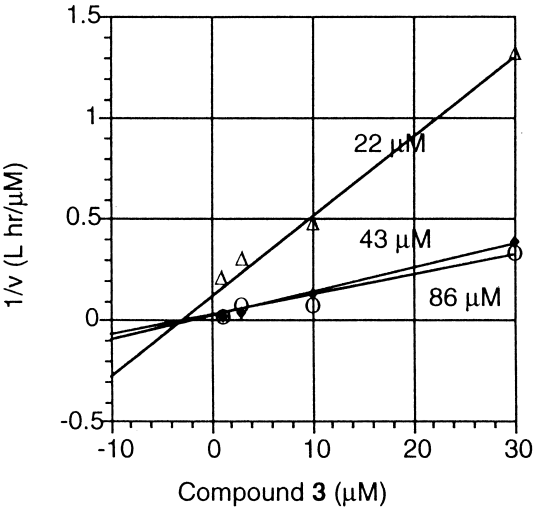

Fig. 3. Dixon plot illustrating the inhibition of MBP-NS345Ps by compound **3**. MBP-NS345Ps 80 μg/ml in a buffer consisting of 50 mM Tris-HCl (pH 7.8), 5 mM $CaCl_2$, 10 mM DTT and 30 mM NaCl was preincubated with compound **3** dissolved in DMSO for 15 min at 37°C. The reaction was started by addition of Pep5 solution at a final concentration of 22 (△), 43 (◆) and 86 μM (○) (a final DMSO concentration of 2%). The reaction was prolonged for 10 min and then quenched by addition of 5 N NaOH. The reaction mixture was analyzed by HPLC.

Several attempts to seek HCV serine proteinase inhibitors have been reported recently including mutated NS4A fragments [12] and detergents such as *n*-octyl-glucoside and

Table 2
Effect of the compounds selected from the chemical library on some serine proteinases

Compound	1	2	3
Chymotrypsin	103.7 ± 4.1[a]	100.9 ± 8.9	100.9 ± 2.3
Factor Xa	47.0 ± 10.6	85.3 ± 5.1	90.5 ± 17.9
Kallikrein	68.2 ± 1.9	87.5 ± 6.0	84.7 ± 6.7
Plasmin	74.8 ± 3.5	83.2 ± 0.8	92.1 ± 3.4
α-Thrombin	96.3 ± 5.5	97.5 ± 5.6	105.1 ± 7.0
Trypsin	26.7 ± 5.2	97.6 ± 2.5	97.6 ± 2.5

[a]The compounds at a concentration of 10^{-5} M were used for the examination. The values of production of the cleavage product relative to the control are expressed as the means ± standard deviations of 3–6 assays.

CHAMP [13], which were supposed to prevent binding of NS3 and NS4A. Variable domain antibody fragments [14,15] as well as nucleic acid fragments [16] showed specific affinity for HCV serine proteinase and were inhibitory for the cleavage activity. Competitive peptidyl inhibitors based upon the NS5A-5B junction were proposed [17]. However, those compounds were relatively large in molecular weight. Furthermore, the detergents were not specific, and the peptides and RNA fragments would be easily subjected to hydrolysis. The compounds sorted out by the assay were non-peptide compounds with low molecular weight and will be prototypes for the further development of anti-HCV agents.

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