Selective Nonpeptidic Inhibitors of Herpes Simplex Virus Type 1 and Human Cytomegalovirus Proteases

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The proteases encoded by herpesviruses including herpes simplex virus type 1 (HSV-1) and human cytomegalovirus (HCMV) are attractive targets for antiviral drug development because of their important roles in viral replication. We randomly screened a chemical compound library for inhibitory activity against HSV-1 protease. 1,4-Dihydroxynaphthalene and three naphthoquinones were found to be potent inhibitors of HSV-1 protease with IC_{50} values of 6.4 to 16.9 μ M. Inhibitory mode analysis of the compounds against HSV-1 protease suggested that, in spite of structural similarities, only 1,4-dihydroxynaphthalene was a competitive inhibitor, whereas the three naphthoquinones were noncompetitive inhibitors. Among all assayed dihydroxynaphthalene derivatives in the chemical compound library, 1,4-dihydroxynaphthalene proved to be the most potent inhibitor of HSV-1 protease. Therefore, the two hydroxyl groups located at positions 1 and 4 on the naphthalene structure seemed essential for exertion of a potent inhibitory activity against HSV-1 protease. In addition, we have found that these compounds are also potent inhibitors of HCMV protease with extremely low micromolar IC_{50} values. This differed from the results of inhibitory mode analysis of HSV-1 protease, 1,4-dihydroxynaphthalene was a noncompetitive inhibitor of HCMV protease, and three naphthoquinones were competitive inhibitors. These compounds showed no effective inhibitory activity against several mammalian serine proteases (trypsin, chymotrypsin, kallikrein, plasmin, thrombin and Factor Xa) at 100 μ M. These results suggest that 1,4-dihydroxynaphthalene and three naphthoquinones may be useful in the development of nonpeptidic antiherpesvirus agents.

Key words HSV-1 protease; HCMV protease; selective inhibitor; dihydroxynaphthalene; naphthoquinone; competitive inhibitor

Herpes simplex virus type 1 (HSV-1) and human cytomegalovirus (HCMV) belong to the family *Herpesviridae* and cause severe opportunistic infections in immunocompromised individuals, including organ transplant recipients and AIDS patients.^{1–3} Present clinical therapies commonly use viral DNA polymerase inhibitors such as acyclovir and ganciclovir, but this approach has been accompanied by the emergence of resistant mutants and various toxicities.¹ Therefore, treatment clinics need new antiviral drugs for combating herpesvirus infections.

Herpesviruses, including HSV-1 and HCMV, encode a protease that is essential for the production of infectious virus particles.^{4,5} The proofs obtained by site-directed mutagenesis^{6,7} and chemical modification with diisopropyl-fluorophosphate^{8–10} indicate that this protease is a serine protease. Recent results for the crystal structures of the HCMV,^{11–14} varicella-zoster virus,¹⁵ HSV-1¹⁶ and HSV-2¹⁶ proteases have shown that these proteases exhibit a novel fold and possess a unique catalytic triad in which the third member of the triad has a histidine substituted for an aspartic acid residue. Since their discovery, herpesvirus proteases have become a novel target of antiviral drugs.

In this paper, we report the characterization of several potent inhibitors of HSV-1 and HCMV proteases obtained by random screening of a chemical compound library with the reverse-phase high-performance liquid chromatography (HPLC) assay system.

MATERIALS AND METHODS

Construction of HSV-1 Protease Expression Vector

The plasmid pRB4057¹⁷ possessing the UL26 gene of HSV-1 was used as the template for polymerase chain reaction

(PCR) amplification of the coding sequence for the protease catalytic domain (amino acids 1–247). Oligonucleotide primers were synthesized based on the published UL26 open reading frame.¹⁸ PCR was performed using Pwo DNA polymerase (Boehringer Mannheim) for 30 cycles of 2 min at 95 °C, 2 min at 68 °C and 2 min at 72 °C. The resulting amplification product was digested with *Eco*RI and *Sma*I before ligation into corresponding sites of the pGEX-4T-1 (Pharmacia Biotech) plasmid. The resulting plasmid pGSTThHP was introduced into *Escherichia coli* BL21(DE3) pLysS to express HSV-1 protease fused in frame to glutathione *S*-transferase.

Expression and Purification of HSV-1 Protease The bacterial cells harboring pGSTThHP were cultured in 1 l of 2×YT medium (1.6% Bacto tryptone, 1% Bacto yeast extract, 0.5% NaCl) containing 100 μ g/ml ampicillin at 30 °C to an OD_{660} of 4, at which time isopropyl-1-thio- β -D-galactopyranoside was added to a final concentration of 0.1 mM, then growth was continued for 14 h. We then resuspended 11 g (wet weight) of the bacterial cell pellet in 55 ml of 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA, lysed the cells by sonication, and ultracentrifuged them at 180000×*g* for 30 min at 4 °C. This supernatant was loaded on a glutathione Sepharose 4B column (Pharmacia Biotech), which was equilibrated with 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, followed by washing and elution with equilibration buffer containing 20 mM reduced glutathione. The fractions containing the protease were pooled, and then dialyzed against 50 mM Tris–HCl, pH 7.5, 100 mM NaCl. The dialyzed pool was concentrated, glycerol of the same volume added, and the mixture stored at –80 °C until use. Protein concentration was determined using a BCA protein assay reagent (Pierce).

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Inhibition Assay for HSV-1 Protease Activity The synthetic substrate TYLQASEKFKMWG-NH₂ was based on the R-site sequence of HSV-1 protease¹⁹ and synthesized by the Peptide Institute Co. (Osaka, Japan). HSV-1 proteases were preincubated with dimethyl sulfoxide (DMSO) as a control, or with the inhibitors dissolved in DMSO at various concentrations for 30 min at 30 °C. The reactions were started by addition of the synthetic substrate and incubated for 1 h at 30 °C, so the final reaction mixtures were composed of 100 μ l of 10% glycerol, 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2% DMSO, 0.25 μ M HSV-1 protease and 500 μ M synthetic substrate. Reactions were stopped by addition of trifluoroacetic acid (TFA) to achieve a final concentration of 1% and the mixtures were subsequently analyzed by HPLC on a 4.6 $\times$ 75 mm ODS-80T_s column (Tosoh, Tokyo), with isocratic elution using 20% acetonitrile in 0.1% TFA. The C-terminal products were detected at 280 nm and quantified by integration of the chromatogram with respect to the standard product SEKFKMWG-NH₂. The percentage of HSV-1 protease inhibitions was determined in comparison with the control.

Construction of HCMV Protease Expression Vector HCMV strain AD169 viral DNA²⁰ containing the UL80 open reading frame was purchased from Advanced Biotechnologies Incorporated. This was used as the template for PCR with the oligonucleotide primers to incorporate an *Eco*RI site and 6 histidine codons at the 5'-end and a *Hind*III site at the 3'-end. Amplification was carried out with the Taq DNA polymerase (Takara, Tokyo) for 25 cycles of 30 s at 95 °C, 45 s at 55 °C and 1 min 30 s at 72 °C. The amplified DNA was cloned into pT7Blue(R) (Novagen) for TA-cloning. To avoid cleavage at the two I-sites of HCMV protease, substitutions of glycine for valine at the P3 position²¹ were introduced using a Transformer Site-Directed Mutagenesis Kit (Clontech). The expression vector pMT1, constructed by replacing the *tac* promoter of pMK2²² with a *trp* promoter, was digested with *Eco*RI and *Hind*III. The mutated HCMV protease gene was inserted into the linearized pMT1. For expression of the HCMV protease, the complete expression vector pMTHHCMVM2 was transferred into *E. coli* TG1.

Expression and Purification of HCMV Protease The bacterial cells retaining pMTHHCMVM2 were cultured in 1 l of 2 $\times$ YT medium containing 100 μ g/ml ampicillin at 37 °C for 18 h. Six and half grams (wet weight) of bacterial cell pellets were resuspended in 33 ml of 20 mM imidazole, 50 mM sodium phosphate, pH 7.4, 300 mM NaCl, and then disrupted by sonication, and ultracentrifuged at 180000 $\times g$ for 30 min at 4 °C. The soluble fraction was applied to a nickel-nitrilotriacetic acid-agarose column (Qiagen), which was equilibrated with 20 mM imidazole, 50 mM sodium phosphate, pH 7.4, 300 mM NaCl. Subsequently, the column was washed with equilibration buffer and the protease was eluted with 200 mM imidazole, 50 mM sodium phosphate, pH 7.4, and 300 mM NaCl. The fractions containing the protease were pooled, concentrated and loaded on a Sephadex G-25 medium column (Pharmacia Biotech) equilibrated with 100 mM sodium phosphate, pH 7.4, 300 mM NaCl. The fractions containing the protease were pooled, concentrated, glycerol of the same volume added, and the mixture stored at -80 °C until needed. Protein concentration was decided

using the BCA protein assay reagent (Pierce).

Inhibition Assay of HCMV Protease Activity The synthetic substrate SYVKASVSPE based on the R-site sequence of HCMV protease¹⁹ was synthesized by Sawady Technology Co. (Tokyo). The enzymes were preincubated with DMSO as a control or with the inhibitors dissolved in DMSO at various concentrations for 30 min at 30 °C. After the reactions had been initiated by adding the substrate, the reaction mixtures were incubated for 1 h at 30 °C. The incubation mixtures were composed of 100 μ l of 10% glycerol, 50 mM sodium phosphate, pH 7.4, 150 mM NaCl, 2% DMSO, 1.0 μ M HCMV protease and 500 μ M synthetic substrate. The reactions were quenched by the addition of TFA to achieve a final concentration of 1% and subsequently analyzed by HPLC on a 4.6 $\times$ 75 mm ODS-80T_s column (Tosoh, Tokyo) with isocratic elution with 9.5% acetonitrile in 0.1% TFA. The N-terminal cleavage products were detected at 280 nm and quantified by integration of the chromatograms with reference to the standard product SYVKA. Inhibitory activities associated with the compounds were expressed as percentages of the control.

Mammalian Serine Protease Assay The enzyme inhibition selectivity of each compound was determined against the mammalian serine proteases, trypsin, chymotrypsin, kallikrein, plasmin, thrombin and Factor Xa. The enzyme-compound mixture for each assay was preincubated for 3 min at 37 °C followed by addition of the substrate. After incubation for 5 min at 37 °C, the enzyme reaction was stopped by addition of acetic acid to achieve a final concentration of 0.5%, and enzymatic activity was measured by scanning on a microplate reader with excitation and emission wavelengths of 365 and 450 nm, respectively. The final conditions for each assay were as follows: 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM CaCl₂, 0.033% polyethylene glycol 6000, 0.917% DMSO with [25 μ M *tert*-butoxycarbonyl (Boc)-QAR-4-methylcoumaryl-7-amide (MCA) and 0.2 U/ml trypsin], [25 μ M succinyl-AAPF-MCA and 0.004 U/ml chymotrypsin], [25 μ M PFR-MCA and 0.05 U/ml kallikrein], [25 μ M Boc-VLK-MCA and 0.4 U/ml plasmin] or [25 μ M benzyloxycarbonyl-L-pyroglutamyl-GR-MCA and 0.1 U/ml Factor Xa], 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM CaCl₂, 0.033% bovine serum albumin, 0.917% DMSO, 25 μ M Boc-D(benzyloxy)-PR-MCA and 0.05 U/ml thrombin. Enzyme inhibitions associated with the compounds were expressed as percentages of the control, which contained only the inhibitor solvent.

RESULTS AND DISCUSSION

Inhibition of HSV-1 Protease by 1,4-Dihydroxynaphthalene and Naphthoquinones Using the HPLC assay system, we performed random screening of a chemical compound library at 100 μ M against HSV-1 protease. Compounds exhibiting 100% inhibition are listed in Table 1. The IC₅₀ values of these potent compounds were then determined. Using these data, compounds **1**–**4** shown in the table were identified as effective inhibitors of HSV-1 protease (IC₅₀ < 20 μ M), compound **1** being the most potent inhibitor. Three naphthoquinones also displayed inhibitory activity, although to a lesser degree than compound **1**, in the order **2** > **3** > **4**. These results suggest that replacement of the 5-hydroxyl group or

5,8-dihydroxyl groups on the naphthoquinone structure leads to reduced inhibitory activity against HSV-1 protease.

Inhibitory Mode of 1,4-Dihydroxynaphthalene and Naphthoquinones against HSV-1 Protease To analyze the inhibitory mode of compounds 1–4, substrate titration experiments were performed in the absence and in the presence of different inhibitor concentrations. The dissociation constants K_i (enzyme–inhibitor complex) and K_{ii} (enzyme–substrate–inhibitor complex) were also determined. The

Table 1. Inhibition of HSV-1 Protease by 1,4-Dihydroxynaphthalene and Naphthoquinones

Compound No.	Compound name	Structure	IC ₅₀ (μM)
1	1,4-Dihydroxynaphthalene		6.4
2	1,4-Naphthoquinone		7.7
3	5-Hydroxy-1,4-naphthoquinone		9.3
4	5,8-Dihydroxy-1,4-naphthoquinone		16.9

IC₅₀ for each compound was determined as described in Materials and Methods. At least four different concentrations of each compound were examined to generate IC₅₀.

Lineweaver–Burk double-reciprocal plot of Fig. 1A shows that, despite structural similarities, only compound 1 exhibits competitive inhibition of HSV-1 protease. The K_i and K_{ii} for compound 1 were determined to be 2.6 μM and a very high value. This result suggests that compound 1 binds to the free enzyme in the substrate-binding groove or active site of HSV-1 protease. By contrast, the same experiment carried out with compounds 2–4 clearly showed that these compounds have a noncompetitive mode of inhibition (Figs. 1B, C and D). The K_i and K_{ii} for these compounds were estimated to be: compound 2, 8.8 and 8.8 μM; compound 3, 4.8 and 4.8 μM; and compound 4, 10.2 and 10.3 μM, respectively. These results suggest that these compounds most likely exhibit inhibitory activity by binding to the free enzyme or the enzyme–substrate complex outside of the substrate-binding groove or the active site of HSV-1 protease.

Inhibition of HSV-1 Protease by Dihydroxynaphthalene Derivatives To examine the effect of the dihydroxyl groups of compound 1 on the inhibitory activity against HSV-1 protease, we assessed the related analogs in the chemical compound library (Table 2). Compounds 5–9 are positional isomers of compound 1, and only compound 5 showed remarkable inhibition against HSV-1 protease activity, although its potency was less than that of compound 1. Compounds 6–9 did not markedly influence this activity, and compounds 10 and 11, which are methoxy analogs of compound 1, also did not inhibit HSV-1 protease activity. Therefore, it seems that the two hydroxyl groups introduced to positions 1 and 4 on the naphthalene structure are essential to exert a potent inhibitory activity against the HSV-1 protease, and the two hy-

Fig. 1. Inhibitory Mode of HSV-1 Protease Activity by 1,4-Dihydroxynaphthalene and Naphthoquinones

Protease activity was determined as described in Materials and Methods. (A) Lineweaver–Burk double-reciprocal plot of HSV-1 protease inhibition by compound 1. Substrate titration curves were recorded in the absence of compound 1 (closed circles) and in the presence of 2 μM (open circles); 4 μM (closed triangles); 6 μM (open triangles). (B) Lineweaver–Burk double-reciprocal plot of HSV-1 protease inhibition by compound 2. Substrate titration curves were recorded in the absence of compound 2 (closed circles) and in the presence of 2 μM (open circles); 4 μM (closed triangles); 6 μM (open triangles). (C) Lineweaver–Burk double-reciprocal plot of HSV-1 protease inhibition by compound 3. Substrate titration curves were recorded in the absence of compound 3 (closed circles) and in the presence of 2.5 μM (open circles); 5 μM (closed triangles); 7.5 μM (open triangles). (D) Lineweaver–Burk double-reciprocal plot of HSV-1 protease inhibition by compound 4. Substrate titration curves were recorded in the absence of compound 4 (closed circles) and in the presence of 5 μM (open circles); 10 μM (closed triangles); 15 μM (open triangles).

Table 2. Inhibition of HSV-1 Protease by Dihydroxynaphthalene Derivatives

Compound No.	Compound name	Structure	% Inhibition at 100 μM	IC ₅₀ (μM)
5	1,2-Dihydroxynaphthalene		100	28.8
6	Naphthoresorcinol		1	>100
7	1,5-Dihydroxynaphthalene		2	>100
8	2,3-Dihydroxynaphthalene		15	>100
9	2,7-Dihydroxynaphthalene		6	>100
10	4-Methoxy-1-naphthol		9	>100
11	1,4-Dimethoxynaphthalene		0	>100

IC₅₀ for each compound was determined as described in Materials and Methods. Four different concentrations of each compound were examined to generate IC₅₀.

Table 3. Inhibition of HCMV Protease by 1,4-Dihydroxynaphthalene and Naphthoquinones

Compound No.	IC ₅₀ (μM)
1	1.0
2	1.0
3	0.8
4	0.9

Inhibition assays were performed as described in Materials and Methods. Reactions were run in duplicate, and data are averages. Six different concentrations of each compound were used for calculating IC₅₀.

droyl groups must not be modified with substituents.

Inhibition of HCMV Protease by 1,4-Dihydroxynaphthalene and Naphthoquinones Compounds 1–4, the inhibitors of HSV-1 protease, were also assessed for inhibitory activity against HCMV protease. As shown in Table 3, these compounds demonstrated inhibitory activity against the protease, with IC₅₀ values in the extremely low micromolar concentration. Therefore, these compounds were found to inhibit HCMV protease more potently than HSV-1 protease. Recently, two naphthoquinones isolated from a medicinal plant were reported to exhibit 15- and 170-fold more potent antiviral activity against HCMV *in vitro* than gancyclovir.²³⁾ We speculate that the antiviral activity of these naphthoquinones may have been exhibited inhibition of HCMV protease in HCMV-infected cells. Accordingly, we expect that compounds 2–4 will also show antiviral activity against HCMV.

Inhibitory Mode of 1,4-Dihydroxynaphthalene and Naphthoquinones against HCMV Protease To analyze the inhibitory mode of compounds 1–4 against HCMV protease, substrate titration experiments were performed in the absence and in the presence of different inhibitor concentra-

tions. The dissociation constants K_i and K_{ii} were also determined. The Lineweaver–Burk double-reciprocal plot of Fig. 2A shows that, despite structural similarities, only compound 1 exhibits noncompetitive inhibition of HCMV protease. The K_i and K_{ii} for compound 1 were determined to be 0.83 and 1.0 μM . This suggests that compound 1 most likely exhibits inhibitory activity by binding to the free enzyme or the enzyme–substrate complex outside of the substrate-binding groove or the active site of HCMV protease. By contrast, the same experiment carried out with compounds 2–4 clearly showed that these compounds have a competitive mode of inhibition (Fig. 2B, C and D). The K_i and K_{ii} for these compounds were estimated to be: compound 2, 0.58 μM and a very high value; compound 3, 0.33 μM and a very high value; and compound 4, 0.39 μM and a very high value, respectively. These results suggest that these compounds bind to the free enzyme in the substrate-binding groove or the active site of HCMV protease.

Effects of 1,4-Dihydroxynaphthalene and Naphthoquinones on Mammalian Serine Proteases Table 4 compares the effects of compounds 1–4 against some mammalian serine proteases, including trypsin, chymotrypsin, kallikrein, plasmin, thrombin and factor Xa, at a concentration of 100 μM . Compounds 1–4 did not inhibit these proteases effectively. These results suggest that compounds 1–4 are selective inhibitors of HSV-1 and HCMV proteases because they do not potently inhibit at 100 μM , which corresponds to a 5.9 to 125-fold concentration of the IC₅₀ values against HSV-1 and HCMV proteases.

As stated above, compound 1 and compounds 2–4 showed a contrastive inhibitory mode to HSV-1 and HCMV proteases. Because HSV-1 and HCMV proteases have the same catalytic triad,¹⁶⁾ it seems that these compounds did not

Fig. 2. Inhibitory Mode of HCMV Protease Activity by 1,4-Dihydroxynaphthalene and Naphthoquinones

Protease activity was determined as described in Materials and Methods. (A) Lineweaver-Burk double-reciprocal plot of HCMV protease inhibition by compound 1. Substrate titration curves were recorded in the absence of compound 1 (closed circles) and in the presence of 1 μ M (open circles). (B) Lineweaver-Burk double-reciprocal plot of HCMV protease inhibition by compound 2. Substrate titration curves were recorded in the absence of compound 2 (closed circles) and in the presence of 1 μ M (open circles). (C) Lineweaver-Burk double-reciprocal plot of HCMV protease inhibition by compound 3. Substrate titration curves were recorded in the absence of compound 3 (closed circles) and in the presence of 1 μ M (open circles). (D) Lineweaver-Burk double-reciprocal plot of HCMV protease inhibition by compound 4. Substrate titration curves were recorded in the absence of compound 4 (closed circles) and in the presence of 1 μ M (open circles).

Table 4. Effects of 1,4-Dihydroxynaphthalene and Naphthoquinones against Several Mammalian Serine Proteases

Compound No.	% Inhibition at 100 μ M					
	Trypsin	Chymotrypsin	Kallikrein	Plasmin	Thrombin	Factor Xa
1	14	17	25	11	17	14
2	21	24	21	14	17	16
3	25	28	41	26	29	29
4	29	28	55	28	34	43

Inhibition assays were performed as described in Materials and Methods. Reactions were run in duplicate, and data are averages.

show inhibition by binding with the catalytic triad of those proteases. Therefore, compound 1 might have exhibited competitive inhibition by binding with a unique substrate binding site in HSV-1 protease. On the other hand, compounds 2–4 might have exhibited competitive inhibition by binding with a unique substrate binding site in HCMV protease.

Several investigations to seek herpesvirus protease inhibitors have recently reported various low molecular weight inhibitors^{24–33)} and peptidomimetic inhibitors.^{21,34,35)} While a low molecular weight inhibitor with *in vitro* antiviral activity and stability in human plasma has been found,²⁹⁾ there are likely to be problems involved with the peptidomimetic inhibitors, which display poor cell penetration and are easily subjected to degradation in cell culture.^{21,35)} These facts suggest that the low molecular weight inhibitors, compounds 1–4 are more suitable than the peptidomimetic inhibitors as antiherpesvirus agents.

In conclusion, 1,4-dihydroxynaphthalene and three naphthoquinones were shown to exhibit low micromolar potency

and selective inhibition against both HSV-1 and HCMV proteases. Moreover, these compounds were found to be an ideal low-molecular-weight competitive inhibitor of HSV-1 or HCMV protease and thus may be useful for development into a nonpeptidic antiherpesvirus drug.

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