BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 643–647 (1997)
ARTICLE NO. RC977358

Novel Hepatitis C Virus Protease Inhibitors: Thiazolidine Derivatives

Kenji Sudo,*,†,[1] Yukiharu Matsumoto,* Masaaki Matsushima,* Masatoshi Fujiwara,* Kenji Konno,* Kunitada Shimotohno,‡ Shiro Shigeta,† and Tomoyuki Yokota*

**Rational Drug Design Laboratories, 4-1-1, Misato, Matsukawa-Machi, Fukushima 960-12, Japan; ‡Institute for Virus Research, Kyoto University, Sakyo-ku Shogoin, Kyoto 606, Japan; and †Department of Microbiology, Fukushima Medical College, 1 Hikarigaoka, Fukushima 960-12, Japan*

Received August 14, 1997

This study evaluated the inhibitory effects of thiazolidine derivatives on hepatitis C virus (HCV) protease and other human serine proteases. The inhibition efficacy was tested with a reversed-phase high-performance liquid chromatography (HPLC) assay system using a NS3-NS4A fusion protein as the HCV protease and a synthetic peptide substrate that mimics the NS5A-5B junction. Nine thiazolidine derivatives showed more than 50% inhibition at 50 μg/ml. The most potent derivative was RD4-6250, with 50% inhibition at a concentration of 2.3 μg/ml; this concentration was lower than those of other protease inhibitors reported previously. The most selective derivative was RD4-6205, with 50% inhibition at a concentration of 6.4 μg/ml, a lower concentration than those on other serine proteases (chymotrypsin, trypsin, plasmin, and elastase). These results suggest that the RD4-6205 skeleton is an important structure for inhibitory activity on the HCV protease NS3-NS4A.

Hepatitis C virus (HCV) is a positive-strand RNA virus (1) and has been classified into its own genus within the family *Flaviviridae* (2). The HCV protein NS3 displays serine protease-like enzymatic activity in the cleavage of the NS3-4A, NS4A-4B, NS4B-5A, and NS5A-5B junctions (3 - 8). The HCV protein NS4A acts as an effector or cofactor in the protease activity of NS3. Shimizu et al. used a synthetic peptide substrate that mimics the NS5A-5B junction to demonstrate that residues 22 - 31 of NS4A are required for its effector activity (9). The proteolytic processing steps in the HCV life cycle are required to produce infectious virus particles; this makes viral proteases primary targets for antiviral agents. Moreover, a refined crystal structures of the HCV NS3 proteinase from HCV BK strain and the HCV NS3 protease domain from HCV H strain complexed with a synthetic NS4A cofactor peptide were determined by X-ray crystallography (10, 11). This information will stimulate efforts to develop more effective anti-HCV therapies.

Recently, we established an in vitro assay system for screening for inhibitors of the NS3-NS4A protease, using reversed-phase high-performance liquid chromatography (HPLC) and a synthetic peptide substrate that mimics the NS5A-5B junction (12). The inhibitory effects of serine protease inhibitors on this HCV protease have been reported, however, they were effective at high concentrations (12). In a present study, we introduced novel HCV protease inhibitors, thiazolidine derivatives, using the established HPLC system. Furthermore, we evaluated the inhibitory effects of these inhibitors on other serine proteases and their cytotoxicities in several cell lines.

MATERIALS AND METHODS

Materials

The 20-mer peptide Dansyl-Gly-Glu-Ala-Gly-Asp-Asp-Ile-Val-Pro-Cys-Ser-Met-Ser-Tyr-Thr-Trp-Thr-Gly-Ala-Leu-OH, which mimics the NS5A-5B junction, was synthesized on an automated multiple peptide synthesizer (Model PSSM-8, Shimazu, Kyoto, Japan). The peptide was purified by preparative reversed-phase HPLC (ODS-80Tm, 2.15 × 30 cm, Tosoh, Tokyo, Japan). Its purity was 92.7%.

The region of the HCV genome encoding NS3- NS4A for expression in *E. coli.* was selected for the active enzyme. This genomic region spans nucleotides 3421 - 5475 (amino acids 1027 - 1711). We expressed the NS3-4A fusion protein as a complex with maltose-binding protein (MBP), which has an affinity for specific ligands that assist enzyme purification, as described previously (12).

Fluorogenic peptides were used as specific substrate for the quantitative measurement of serine proteases, as described previously (13-16). The following fluorogenic peptides were purchased from Peptide Institute, Inc., Osaka, Japan: 3114-v (Suc-Ala-Ala-Pro-Phe-MCA {4-methylcoumaryl-7-amide}), specific for chymotrypsin; 3092-v (Bz-Arg-MCA), specific for trypsin; 3105-v (Boc-Gln-Lys-Lys-MCA), specific for plasmin; and 3100-v (Suc-Ala-Pro-Ala-MCA), specific for elas-

[1] Corresponding author. Fax: + 81-245-67-5554.

0006-291X/97 $25.00

tase. Chymotrypsin and trypsin were purchased from Funakoshi Co., Tokyo, Japan. Plasmin, elastase, and aprotinin were purchased from Sigma Chemical Company, St. Louis, MO, USA. Elastinal was purchased from Peptide Institute, Inc., Osaka, Japan. Chymostatin was purchased from Genosys Bio Technologies, Inc., Cambridge, UK.

The following cells were maintained and used for a cytotoxicity assay. Madine Darby canine (MDCK) cells and MRC-5 cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 8% heat-inactivated fetal calf serum (FCS), 100 units/ml of penicillin G, and 100 μg/ml of streptomycin. HeLa cells were maintained in MEM supplemented with 8% heat-inactivated FCS, 1.6% glucose, and antibiotics. MT-4 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 1.6% glucose, and antibiotics.

Methods

HCV NS3-NS4A assay. Protease activity was determined in a total volume of 200 μl of assay buffer [50 mM Tris-HCl, pH 7.6, containing 30 mM NaCl, 1 mM $CaCl_2$, and 2 mM ($\pm$)-dithiothreitol (DTT)] containing 1.4 μg of MBP-NS3-NS4A (at a final concentration of 0.0603 μM) and 12 μg of the synthetic peptide (at a final concentration of 26 μM). The reaction temperature was 37°C. After an adequate incubation period, the reaction was stopped by adding 4 μl of 2.5 M acetic acid; the reaction mixture was then transferred to a vial for HPLC analysis. Analytical HPLC was carried out using a Tosoh SC-8020 liquid chromatography system (Tokyo, Japan), complete with a binary solvent delivery system, a heated column compartment, and an auto-injection system. The HPLC conditions were as follows: ODS-80Ts column, 0.46 $\times$ 15 cm, Tosoh, Tokyo; Buffer D, 0.1% trifluoroacetic acid (TFA) in water; Buffer E, 0.09% TFA in 90% acetonitrile; 0-50% Buffer D gradient over a 20 min period at a flow rate of 1.0 ml/min. Peptides were detected by UV absorbance at 214 nm. The cleavage products, product A (Dansyl-Gly-Glu-Ala-Gly-Asp-Asp-Ile-Val-Pro-Cys) and product B (Ser-Met-Ser-Tyr-Thr-Trp-Thr-Gly-Ala-Leu-OH), were quantified from their peak heights.

Inhibition of NS3-NS4A protease activity. The MBP-NS3-NS4A was pretreated with inhibitors (at a final concentration of 100 μg/ml) for 15 min at 37°C. Then, the proteolytic reactions were initiated by the addition of the synthetic substrate described above and incubated for 1 h at 37°C. The percentage of NS3-NS4A inhibition was calculated from the peak height of product A.

Serine protease assay. The fluorogenic assay was performed in phosphate-buffered saline using 96-well microtiter trays (MS-8596, Sumitomo Bakelite Co., Ltd., Tokyo, Japan). Serial 5-fold dilutions of the inhibitors were made directly in the microtiter trays using an 8-channel pipette. In a final volume of 200μl, peptidyl-MCA substrates (at a final concentration of 0.1 mM) were mixed with chymotrypsin (0.001 mg/ml), trypsin (0.1 mg/ml), plasmin (0.1 mg/ml), or elastase (1 mg/ml). The plates were incubated for 1 h at 37°C. Enzymatic activity was measured by fluorophotometrical determination (excitation wavelength, 380 nm; emission wavelength, 460 nm) using Fluostar (Tecan Japan Co., Tokyo, Japan).

Cytotoxicity assay. HeLa cell and MT-4 cell suspension were added to each well of a 96-well microtiter tray containing various concentrations of the test compound. MDCK cells and MRC-5 cells were seeded into a 96-well microtiter tray and incubated at 37°C in 5% CO_2. When the cell cultures were confluent, the test compound was added. After a 5 or 6 day incubation, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method. The 50% cytotoxic concentration (CC_{50}) of the test compound was defined as the concentration that reduced the absorbance of the cells to 50% of that of the controls.

RESULTS AND DISCUSSION

Thiazolidine derivatives shown in Figure 1 were evaluated by an in vitro assay system for screening inhibitors of the NS3-NS4A protease using HPLC and a synthetic peptide substrate that mimics the NS5A-5B junction. Shimizu et al. demonstrated that the coefficient for proteolytic efficiency of the NS3 protease expressed in *E. coli.* was increased about 70 times by addition of a 50-fold molar excess of NS4A fragment (9). We compared the effects of NS4A fragment (4A18-40), derived from residues 18-40 of NS4A, on NS3-NS4A and NS3 protease activities in the presence of 50-fold molar excess of 4A18-40, which the maximum enhancement of the proteolytic cleavage was observed at the molar ratio. The proteolytic activity of NS3 protease for synthetic substrate increased with 4A18-40. The rate of substrate cleavage by NS3-NS4A was significantly faster than that by NS3 alone, however, was slow as compared to NS3 with 50-fold molar excess of 4A18-40. Moreover, the rate of cleavage by NS3-NS4A was accelerated in the presence of 4A18-40, that was equal to that by NS3 with 4A18-40 (data not shown). It suggests that NS3 protease activity of the MBP-NS3-NS4A was enhanced by 4A18-40, and incomplete molecules as protease for lacking of NS4A remain in the NS3-NS4A complex expressed in *E. coli.*

The inhibition of NS3-NS4A protease activity by thiazolidine derivatives was measured and incapacitating concentration (IC_{50}) values were calculated; the results are summarized in Table 1. Nine of the fifteen derivatives assayed produced more than 50% inhibition at 50 μg/ml. The following derivatives, listed with their IC50 values, were most significant: RD4-6250, 2.3 μg/ml; RD4-6205, 6.4 μg/ml; RD4-6193, 6.5 μg/ml; RD4-6204, 9.7 μg/ml. Recently, the efficacy of several potent protease inhibitors on HCV protease activity were evaluated using the HPLC method and the *trans*-cleavage assay using in vitro transcription and translation (12, 17-19). Three serine protease inhibitors showed at least 50% inhibition of NS3-NS4A protease activity at concentrations higher than those of the thiazolidine derivatives. These serine protease inhibitors, listed with their IC_{50} values, were: N-Tosyl-L-lysyl chloromethyl ketone (TLCK), 55.4 μg/ml; chymostatin, 344.8 μg/ml; and diisopropyl fluorophosphate (DFP), 415.9 μg/ml (12). Other reports suggest that serine protease inhibitors do not consistently inhibit the HCV protease (18, 19); yet nine thiazolidine derivatives inhibited HCV protease activity at lower concentrations.

Table 1 also shows the inhibitory effect of thiazolidine derivatives against four other serine proteases. The derivatives that strongly inhibited HCV protease also had inhibitory effects on the other serine proteases. Almost all of the thiazolidine derivatives showed 50% inhibition of at least one serine protease at lower concentrations than those of derivatives inhibiting the NS3-NS4A protease.

A protease assay was performed based on the following IC_{50} values: chymostatin, which specifically inhibits chymotrypsin, 0.0081 μg/ml; aprotinin, which specifi-

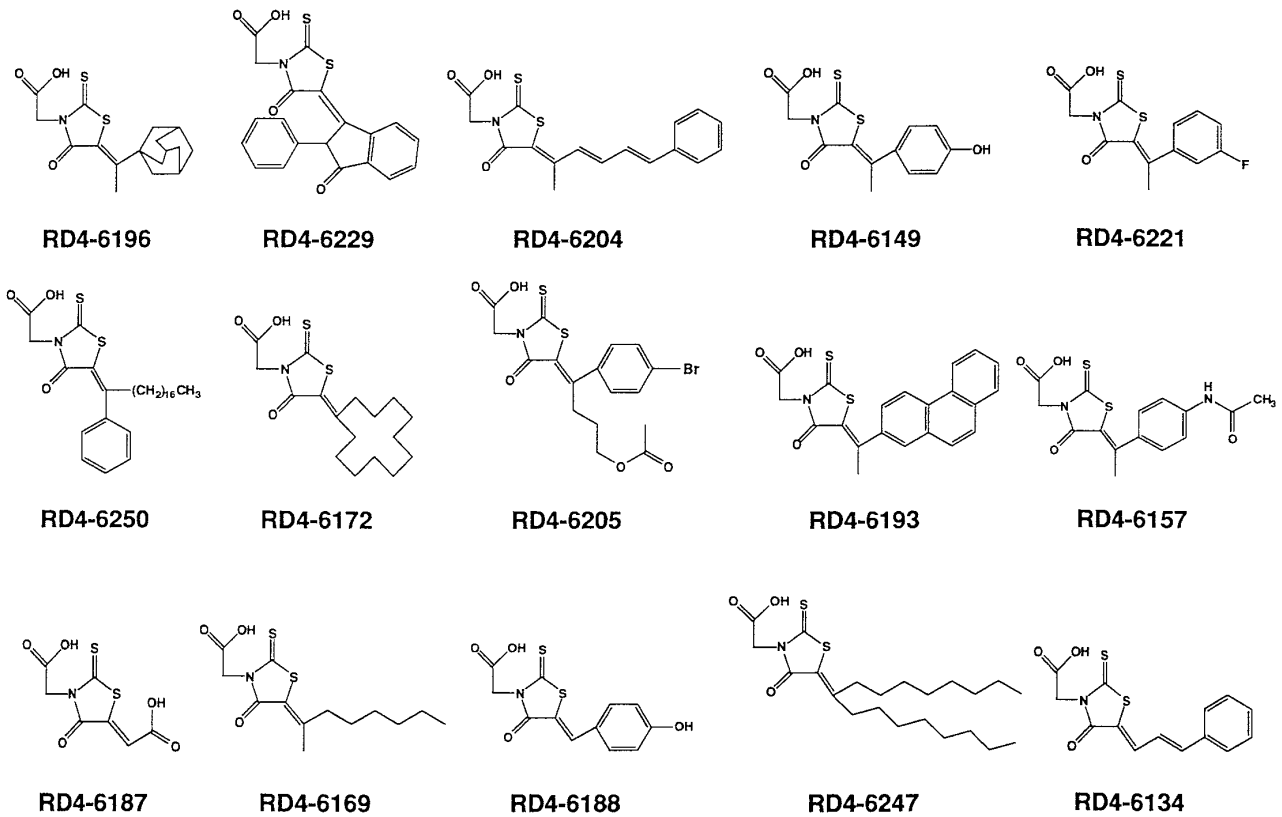

FIG. 1. Structural formula of thiazolidine derivatives tested.

cally inhibit trypsin, 3.58 μg/ml; aprotinin, which specifically inhibit plasmin, 1.20 μg/ml; d elastinal, which specifically inhibit trypsin, 2.64 μg/ml. The thiazolidine derivative RD4-6250, which most strongly inhibited HCV protease, inhibited chymotrypsin and trypsin at lower concentrations than that at which it inhibited the HCV protease NS3-NS4A. Similarly, the derivative RD4-6193 inhibited plasmin at a lower concentration than that at which it inhibited HCV protease. However, the derivatives RD4-6204 and RD4-6205 inhibited the

TABLE 1

Inhibitory Activity of Thiazolidine Derivatives against NS3-NS4A Protease and Several Serine Proteases (IC50:μg/ml)

	Proteases				
Derivatives	HCV	Chymotrypsin	Trypsin	Plasmin	Elastase
RD4-6196	>50	>50	>50	>50	>50
RD4-6229	23.5	9.69	17.3	>50	>50
RD4-6204	9.7	10.8	12.6	>50	21.6
RD4-6149	45.9	21.5	16.9	26.3	35.3
RD4-6250	2.3	0.79	0.73	>50	5.85
RD4-6172	>50	>50	>50	>50	>50
RD4-6205	6.4	15.6	18.3	>50	>50
RD4-6193	6.5	14.5	10.0	2.2	31.9
RD4-6187	>50	>50	>50	>50	>50
RD4-6221	>50	>50	45.1	>50	>50
RD4-6134	>50	3.7	5.8	19.6	24.1
RD4-6157	48.0	3.6	18.0	23.1	38.9
RD4-6169	49.9	0.52	20.9	27.4	>50
RD4-6188	>50	5.4	12.9	16.6	20.4
RD4-6247	14.2	<0.4	3.7	4.6	19.2

TABLE 2

Cytotoxicity of Thiazolidine Derivatives against Several Cell Lines (IC50:μg/ml)

	Cells			
Derivatives	MDCK	HeLa	MT-4	MRC-5
RD4-6196	50.0	10.3	32.1	>50
RD4-6229	>50	>50	9.7	23.1
RD4-6204	20.9	16.3	19.2	23.3
RD4-6149	>50	30.2	5.0	>50
RD4-6250	15.6	16.7	4.5	4.2
RD4-6172	20.4	17.7	21.7	22.4
RD4-6205	50.0	16.9	22.6	22.4
RD4-6193	20.9	10.6	18.5	14.9
RD4-6187	50.5	14.3	30.6	>50
RD4-6221	>50	37.5	4.6	19.6
RD4-6134	>50	50.0	22.5	>50
RD4-6157	>50	18.9	>50	>50
RD4-6169	26.2	10.9	>50	23.1
RD4-6188	50.0	27.4	41.0	>50
RD4-6247	13.4	16.7	19.2	4.5

HCV protease at lower concentrations than those at which they inhibited the four other serine proteases. The selectivity indexes (IC_{50} value against NS3-NS4A protease/IC_{50} value against other serine protease) of RD4-6205 are especially significant: 2.4 against chymotrypsin, 2.9 against trypsin, > 7.8 against plasmin, and > 7.8 against elastase.

Table 2 shows the 50% cytotoxic concentration (CC_{50}) of thiazolidine derivatives in several cell lines. Almost all of the derivatives indicated 50% cytotoxicities at less than 50 μg/ml. The CC_{50} values of RD4-6204, RD4-6250, RD4-6205, and RD4-6193 against all tested cell lines were higher than their IC_{50} values of HCV protease inhibition. The selectivity indexes of RD4-6205 were 7.8 against MDCK cells, 2.6 against HeLa cells, 3.5 against MT-4 cells, and 3.5 against MRC-5 cells. These results show that RD4-6205 is the most selective compound of the derivatives.

The thiazolidine derivative 5-(alpha-heptadecylbenzylidene)-4-oxo-2-thioxo-3-thiazolidine acetic acid (named RD4-6250) shows the strongest HCV protease inhibition. Similarly, when R is 1-octylnonylidene (in the derivative named RD4-6247), the IC_{50} value against HCV protease is 14.2 μg/ml. However, both compounds with the long chain have no specificity for NS3-NS4A protease. On the other hand, the derivative with 5-(1-methyl-5-phenyl)-2,4-pentadienylidene (named RD4-6204) is a more selective inhibitor. The derivative with 5-cinnamylidene (named RD4-6134) shows no inhibition of NS3-NS4A protease. The most selective inhibitor is 4-oxo-5-(5-oxo-1,5-diphenyl-3-phenyl)-2-thioxo-3-thiazolidine-acetic acid (named RD4-6205).

The mode of RD4-6205 inhibition of NS3-NS4A protease activity was examined, and the results shown as a Lineweaver-Burk plot in Figure 2. The Michaelis constant, Km, was calculated to be 68.8 μM, consistent with that determined previously (12). Moreover, the inhibition constants Ki (inhibition of free enzyme) and Kii (inhibition of enzyme-substrate complex) were also determined to be 5.3 and 5.5 μM, respectively. This kinetic analysis revealed that RD4-6205 inhibits the HCV protease in a noncompetitive manner, and thus interacts with both the enzyme and the enzyme-substrate complex.

Most of the thiazolidine derivatives tested displayed a broad range of effects, inhibiting NS3-NS4A protease activity along with the activities of chymotrypsin, trypsin, plasmin, and elastase. This lack of specificity and the degree of inhibition against human serine protease make thiazolidine derivatives themselves inappropri-

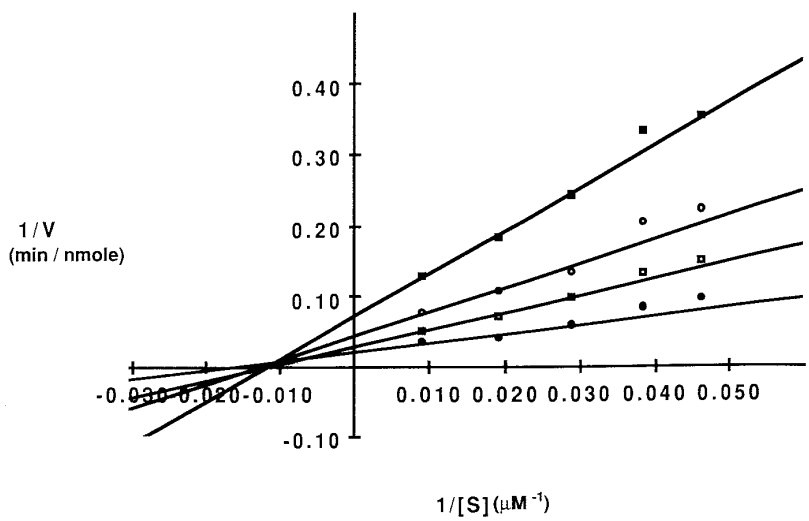

FIG. 2. Lineweaver–Burk plot for inhibition of NS3-NS4A protease activity by RD4-6205. Inhibitor concentration, μg/ml:(■) 20, (○) 10, (□) 5, (●) 0.

ate candidates for clinical use, with several possible exceptions. However, the selective inhibition of HCV protease activity by RD4-6204 and RD4-6205 suggests a protease inactivators design that incorporates structural features of thiazolidine.

ACKNOWLEDGMENTS

We are most grateful to Ms. N. Yamaguchi and Ms. S. Yamada for their excellent technical assistance.

REFERENCES

1. Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W., and Houghton, M. (1989) *Science* **244,** 359–362.
2. Wengler, G. (1991) *Arch. Virol.* **2s,** 223–233.
3. Bartenschlager, R., Ahlborn-Laake, L., Mous, J., and Jacobsen, H. (1993) *J. Virol.* **67,** 3835–3844.
4. Eckart, M. R., Selby, M., Masiarz, F., Lee, C., Berger, K., Crawford, K., Kuo, C., Kuo, G., Houghton, M., and Choo, Q. L. (1993) *Biochem. Biophys. Res. Commun.* **192,** 399–406.
5. Grakoui, A. D., McCourt, D. W., Wychowcki, C., Feinstone, S. M., and Rice, C. M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10583–10587.
6. Grakoui, A. D., McCourt, D. W., Wychowcki, C., Feinstone, S. M., and Rice, C. M. (1993) *J. Virol.* **67,** 2832–2843.
7. Hijikata, M., Mizushima, H., Akagi, T., Mori, S., Kakiuchi, N., Kato, N., Tanaka, T., Kimura, K., and Shimotohno, K. (1993) *J. Virol.* **67,** 4665–4675.
8. Tomei, L., Failla, C., Santolini, E., De Francesco, R., and La Monica, N. (1993) *J. Virol.* **67,** 4017–4026.
9. Shimizu, Y., Yamaji, K., Masuho, Y., Yokota, T., Inoue, H., Sudo, K., Satoh, S., and Shimotohno, K. (1996) *J. Virol.* **70,** 127–132.
10. Love, R. A., Parge, H. E., Wickersham, J. A., Hostomsky, Z., Habuka, N., Moomaw, E. W., Adachi, T., and Hostomska, Z. (1996) *Cell* **87,** 331–342.
11. Kim, J. L., Morgenstern, K. A., Lin, C., Fox, T., Dwyer, M. D., Landro, J. A., Chambers, S. P., Markland, W., Lepre, C. A., O'Malley, E. T., Harbeson, S. L., Rice, C. M., Murcko, M. A., Caron, P. R., and Thomson, J. A. (1996) *Cell* **87,** 343–355.
12. Sudo, K., Inoue, H., Shimizu, Y., Yamaji, K., Konno, K., Shigeta, S., Kaneko, T., Yokota, T., and Shimotohno, K. (1996) *Antiviral Res.* **32,** 9–18.
13. Sawada, H., Yokosawa, H., Hoshi, M., and Ishii, S. (1983) *Experientia* **39,** 377–378.
14. Oshima, G., Akashi, K., and Yamada, M. (1984) *Arch. Biochem. Biophys.* **233,** 212–218.
15. Kanaoka, Y., Takahashi, T., Nakayama, K., Takada, K., Kimura, T., and Sakakibara, S. (1977) *Chem. Pharm. Bull.* **25,** 3126–3128.
16. Kato, H., Adachi, N., Ohno, Y., Iwanaga, S., Takada, K., and Sakakibara, S. (1980) *J. Biochem.* **88,** 183–190.
17. Hahm, B., Han, D. S., Back, S. H., Song, O-K., Cho, M-J., Kim, C-J., Shimotohno, K., and Jang, S. K. (1995) *J. Virol.* **69,** 2534–2539.
18. Lin, C., and Rice, C. M. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7622–7626.
19. D'Souza, E. D. A., Grace, K., Sangar, D. V., Rowlands, D. J., and Clarke, B. E. (1995) *J. Gen. Virol.* **76,** 1729–1736.