Available online at www.sciencedirect.com
ScienceDirect
Antiviral Research 78 (2008) 194–201
Antiviral Research
www.elsevier.com/locate/antiviral

PA subunit of RNA polymerase as a promising target for anti-influenza virus agents

Misako Nakazawa *, Shin-etsu Kadowaki, Izumi Watanabe, Youko Kadowaki, Masaya Takei, Hideyuki Fukuda

Discovery Research Laboratories, Kyorin Pharmaceutical Co. Ltd., Tochigi 329-0114, Japan

Received 23 May 2007; accepted 19 December 2007

Abstract

RNA polymerase of influenza virus is a specific enzyme necessary for the viral replication. A siRNA against the RNA polymerase and the RNA polymerase inhibitor L-742,001 reduced accumulation of viral RNAs in the infected cells. L-742,001 strongly inhibited virus re-growth after removal of the agent from the culture, whereas the neuraminidase inhibitor zanamivir did not. L-742,001-resistant mutants showed a Thr-20 to Ala substitution in the PA subunit of RNA polymerase. The drug-resistant virus showed a slight reduction in the susceptibility to L-742,001 in both the plaque assay (threefold reduction) and enzyme assay (two- to three-fold reduction). The resistance levels were lower than those of zanamivir-resistant mutants in the plaque assay. Against zanamivir-resistant mutants, L-742,001 retained the same antiviral activity as against the wild-type strain. These results indicate that L-742,001 is most likely to act at the PA subunit, and possesses a unique profile. It is suggested that PA subunit of RNA polymerase is a promising target for anti-influenza virus agents.

Keywords: Influenza virus; RNA polymerase; PA subunit

1. Introduction

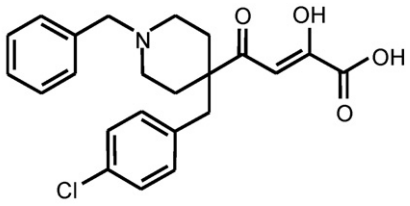
Influenza virus is one of the major causative agents of respiratory viral infections, and carries high rates of morbidity and mortality. Infection with the influenza virus is a life-threatening event for high-risk patients, such as the elderly, residents of nursing homes, and those with cardiovascular, pulmonary, or renal disease, diabetes, and/or immunosuppression. The influenza virus is rapidly disseminated around the world in seasonal epidemics, and sometimes 10–20% of the total population are infected. The World Health Organization reported that annual epidemics resulted in 250,000–500,000 deaths all over the world (World Health Organization, 2004).

Vaccination is the most common prophylaxis. Vaccination can prevent infection in 70–90% of healthy adults, but in only 30–40% of elderly and immunocompromised individuals (Fukuda et al., 1999; Nichol et al., 1998). Therefore, the administration of antiviral agents is an important option for the influenza virus infection. Now, two classes of anti-influenza agents, ion channel blockers and neuraminidase (NA) inhibitors, are used for the treatment of influenza viral infection. The ion channel blockers, amantadine and rimantadine, inhibit virus replication at the early stage of the infection by blocking the M2 ion channel. However, these ion channel blockers show no activity against the influenza B virus and rapidly select resistant virus strains (Hayden and Couch, 1992). In some clinical settings, these agents often caused serious side effects. On the other hand, the NA inhibitors, zanamivir and oseltamivir, are effective against both the influenza A and B viruses with low side effects. Although NA inhibitor-resistant viruses have been isolated in clinical settings as well as in vitro, these resistant viruses are less pathogenic (Carr et al., 2002; Herlocher et al., 2002). The clinical usefulness of NA inhibitors has been reported (Cheer and Wagstaff, 2002; Kaiser et al., 2003).

Recently, some NA-resistant viruses have been reported to be transmissible to humans and ferrets (Herlocher et al., 2004; Kiso et al., 2004). Also, oseltamivir-resistant viruses have been isolated from highly virulent H5N1 avian influenza viruses (Le et al., 2005). In such situation, it is necessary to prepare other

* Corresponding author at: Discovery Research Laboratories, Kyorin Pharmaceutical Co. Ltd., 2399-1 Nogi, Shimotsuga, Tochigi 329-0114, Japan. Tel.: +81 280 56 2201; fax: +81 280 57 1293.
E-mail address: misako.nakazawa@mb.kyorin-pharm.co.jp (M. Nakazawa).

0166-3542/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2007.12.010



IC_{50} (μg/mL)	
Plaque	RNA polymerase
4.86	0.69

Fig. 1. Structure and in vitro activity of L-742,001.

treatment options for influenza virus infection than existing anti-influenza virus agents.

Influenza virus RNA polymerase, which consists of three subunits, PB1, PB2, and PA, is a unique protein because of its multifunctional activities, including capped RNA binding, endonuclease cleavage, and RNA-directed RNA transcription activities. These functions are essential for virus replication. Also RNA polymerase of influenza virus is highly conserved among influenza viruses A, B, and C (Yamashita et al., 1989), but it has no homologues in mammalian cells.

Some researchers have reported the antiviral effect of RNA interference (RNAi) against RNA polymerase using specific small interfering RNA (siRNA). RNA polymerase-specific siRNA inhibited virus production in both in vitro cultured cells and in vivo murine models (Ge et al., 2003, 2004; Tompkins et al., 2004). Also, several compounds have been identified as RNA polymerase inhibitors, i.e., 2,4-diketobutanoic acids (Tomassini et al., 1994; Hastings et al., 1996), flutimide (Tomassini et al., 1996), hydroxamic acids (Cianci et al., 1996), tetramic acids (Parkes et al., 2003), and T-705 (Furuta et al., 2005). Among these compounds, L-742,001, a 2,4-diketobutanoic acid analogue (Fig. 1, Hastings et al., 1996) showed the most potent RNA polymerase inhibition without any cytotoxicity.

In this study, we showed that the PA subunit of RNA polymerase of influenza virus would be a key target for anti-influenza virus agents. Furthermore, we documented the unique antiviral profile of an RNA polymerase inhibitor, L-742,001.

2. Materials and methods

2.1. Virus, cells, and compounds

We used A/PR/8/34 (PR8) as the influenza virus and Madin-Darby canine kidney (MDCK) cells as the host cells. MDCK cells were maintained in minimum essential medium (MEM; Sigma–Aldrich, St. Louis, MO) containing 10% heat-inactivated FCS, 50 units/mL penicillin, and 50 μg/mL streptomycin at 37 °C with 5% CO_2. L-742,001 (Hastings et al., 1996) was synthesized at Kyorin Pharmaceutical Co. Ltd. (Tokyo, Japan). Zanamivir was purchased from a commercial source.

2.2. siRNA introduction

siPA-1, a PA-specific siRNA, was constructed based on PA-2087 (Ge et al., 2003), and synthesized by Ambion Inc. (Austin, TX). The sequence of siPA-1 was as follows: sense, 5′-GCAAUUGAGGAGUGCCUAAdTdT-3′; antisense, 5′-UUAGGCACUCCUCAAUUGCdTdT-3′.

siRNA was introduced to the host cells according to Ge et al. (2003) with minor modifications. Briefly, MDCK cells were trypsinized, washed, and re-suspended in serum-free RPMI 1640 medium (Sigma–Aldrich) at 1×10^7 cells/mL. Cells were mixed with 2.5 nmol/mL siRNA and electroporated at 400 V and 800 μF using a GTE-10 electroporation system (Shimadzu, Kyoto, Japan). Electroporated cells were re-suspended in RPMI 1640 medium containing 10% FCS, plated on six-well plates, and cultured for 24 h. The cells were used for plaque assays and the quantification of intracellular viral RNA accumulation.

2.3. Plaque assays

Confluent MDCK cells in six-well plates were infected with 50–100 PFU of the PR8 virus per well. After 1 h, each well was overlaid with MEM containing 0.6% agarose, 0.2% BSA, 10 mM HEPES buffer, 0.01% DEAE dextran, 0.01 mg/mL acetyl-trypsin, and antiviral agents at several concentrations. The agar overlay was removed after 2 days of incubation at 36 °C with 5% CO_2. Plaques were visualized by staining the cells with 0.1% crystal violet, and the plaques were counted. The drug concentration required for 50% inhibition (50% inhibitory concentration [IC_{50}]) was calculated using linear regression analysis of a dilution series covering the effective concentrations of the drug.

2.4. Quantification of intracellular viral RNA accumulation

Confluent MDCK cells were infected with the PR8 virus. If necessary, MDCK cells infected with the PR8 virus were cultured with the treatment of either siRNA or L-742,001. Several hours after cultivation, the culture medium was removed and the cells were lysed using Trizol LS reagent (Invitrogen, Carlsbad, CA). Viral RNA was isolated from lysates following the Manufacturer's protocol. Reverse transcription (RT) was carried out using an Omniscript reverse transcriptase kit (Qiagen, Valencia, CA) in a 20 μL reaction mixture, containing 200 ng of total RNA and specific primers, at 37 °C for 1 h. The sequences of the specific primers were as follows: Nucleoprotein (NP) mRNA, PA mRNA, and β-actin mRNA, $pd(T)_{12\text{-}18}$ (Amersham Biosciences, Piscataway, NJ); 18S rRNA, random hexamer (Invitrogen); NP virion RNA (vRNA), 5′-CTCATCCTTTATGACAAAGAAG-3′; PA vRNA, 5′-GCTTCTTATCGTTCAAGCTCTTAGG-3′.

One microliter of RT reaction mixture was then used for real-time PCR using gene-specific primers, TaKaRa Ex Taq^R HS (Takara Bio, Shiga, Japan) and SYBR Green I (Invitrogen). The sequences of PCR primers for each RNA are shown as follows: NP vRNA and NP mRNA, 5′-CTCATCCTTTATGACAAAGAAG-3′ and 5′-AGATCATCATGTGAGTCAGAC-3′; PA vRNA and PA

mRNA, 5′-GCTTCTTATCGTTCAAGCTCTTAGG-3′ and 5′-CCAAGAAGCATTAAGCAAAACCCAG-3′; β-actin mRNA (host cell), 5′-ACGGCATCGTCACCAACTG-3′ and 5′-TGTTGAACGTCTCGAACATGATCTG-3′; 18S rRNA (host cell), 5′-CGGCTACCACATCCAAGGAAG-3′ and 5′-AGCTGGAATTACCGCGGCTG-3′.

Before the PCR, the mixture was incubated at 94 °C for 2 min. The reaction was then basically performed at 94 °C for 20 s, 55 °C for 30 s, 72 °C for 30 s, and 75 °C for 6 s for 35 cycles. The amounts of PCR products were monitored with a SmartCycler (Cepheid, Sunnyvale, CA) and analyzed with Cepheid SmartCycler Software (Version 1.2b). Each amount of viral RNA was normalized to the level of β-actin mRNA or 18S rRNA of host cells in the same sample. In this experiment, NP vRNA and NP mRNA were used as representative of the general virus RNA. β-actin mRNA and 18S rRNA were used as the internal standards of the host cells.

2.5. RNA polymerase inhibitory assay

The influenza RNA polymerase inhibitory assay was performed by a modification of a previously described procedure (Bouloy et al., 1978). Briefly, the PR8 virus was grown in 10-day-old embryonated eggs, purified by ultracentrifugation, and re-suspended in 10 mM Tris–HCl (pH 7.8), 100 mM NaCl, 1 mM DTT, and 20% glycerol. Purified PR8 virus (60 ng) was solubilized and incubated for 45 min at 30 °C with various concentrations of the drug solution and reaction buffer (1 μg/mL globin mRNA, 50 mM HEPES–NaOH pH 7.0, 0.1% NP-40, 2.5 mM DTT, 0.5 mM EDTA, 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM ATP, CTP, GTP, 2 μM UTP, and 5 μCi ^{32}P-α-UTP (3000 Ci/mmol, Amersham Biosciences). The reaction product was precipitated by trichloroacetic acid and then filtered through a Whatman GF/C glass fiber filter. The inhibitory activity was quantified using liquid scintillation counting, and then IC_{50} was calculated.

2.6. Isolation of L-742,001- and zanamivir-resistant viruses

L-742,001-resistant PR8 viruses were isolated after in vitro serial passages. MDCK cells infected with the PR8 virus in MEM containing 10 mM HEPES buffer, 0.1% BSA, 1 μg/mL acetyl-trypsin, and L-742,001 were incubated for 3–7 days at 36 °C with 5% CO_2 until a cytopathic effect was observed. The culture supernatants were centrifuged, and aliquots were used for sequential selection. A resistant virus was generated upon six passages (P1–P6) of the parent virus at a MOI of 0.05 after stepwise exposure to the antiviral compounds. Each passage was performed at the following concentrations: 2 (for P1), 4 (for P2), 8 (for P3), 32 (for P4), 32 (for P5), and 32 (for P6) μg/mL. The strains generated by the respective passages were designated as PL-1, PL-2, PL-3, PL-4, PL-5, and PL-6. As a control, the parent PR8 virus was also passaged the same number of times in MDCK cells in the absence of the antiviral agent. The strains isolated by the respective passages were designated as PC-1, PC-2, PC-3, PC-4, PC-5, and PC-6.

Zanamivir-resistant viruses were isolated after four serial passages (P1–P4) with up to 1000 μg/mL of zanamivir in the same way as described above. Each isolated strain was designated as PZ-1, PZ-2, PZ-3, and PZ-4, respectively.

2.7. Characterization of L-742,001- and zanamivir-resistant viruses

PL-1, PL-2, and PL-6 were used as the representatives of L-742,001-passaged strains. The susceptibility of each virus to L-742,001 was measured by a plaque assay. Also, the sequence of cDNA of each virus was analyzed. Viral RNA was isolated from the supernatant of the cells infected with each virus using Trizol LS reagent. Reverse transcription (RT) was carried out with Superscript II RNase H-Reverse Transcriptase (Invitrogen). PCRs of PB1, PB2, PA, and NP genes were performed using pfu Turbo Hotstart DNA polymerase (Stratagene, La Jolla, CA) and gene-specific primers, which were based on PB1, PB2, PA, and NP sequences (Genbank accession numbers AF389116, AF389115, AF389117, and AF389119, respectively). Each PCR-amplified product was purified and sequenced by the dye-terminator method.

PZ-4 strains were used as the representatives of zanamivir-passaged strains. The mutations in the neuraminidase (NA) gene and hemagglutinin (HA) gene of PZ-4 strains were determined using NA and HA gene-specific primers based on the reported gene sequence (Accession number AF389120 and AF389118).

2.8. Re-growth of virus after removal of anti-influenza virus agents

Virus re-growth after removal of the compounds was monitored by the method reported previously (Takahashi et al., 2003). The MDCK cells in 96-well plates were infected with the PR8 virus at a MOI of 0.001. After 30 min, the inoculum was removed, and the cells were overlaid with MEM containing 10 mM HEPES buffer, 0.1% BSA, 1 μg/mL acetyl-trypsin, and 3 and 10 μg/mL of L-742,001 or zanamivir. After 10 h incubation at 35 °C with 5% CO_2, the supernatants were removed, then each well was washed and further incubated with drug-free medium. The virus yields in the supernatant were measured by plaque assay at 20 h post-infection.

3. Results

3.1. Knockdown effect on RNA polymerase by siRNA

The knockdown effect of siRNA siPA-1, specific to the PA subunit of RNA polymerase, was observed as in the case of PA-2087 (Ge et al., 2003). Pre-treatment of MDCK cells with siPA-1 completely inhibited PR8 virus production. siPA-1 strongly down-regulated PA mRNA and PA vRNA by levels exceeding 90% compared with untreated cells (Fig. 2). siPA-1 also inhibited the accumulation of NP mRNA and NP vRNA.

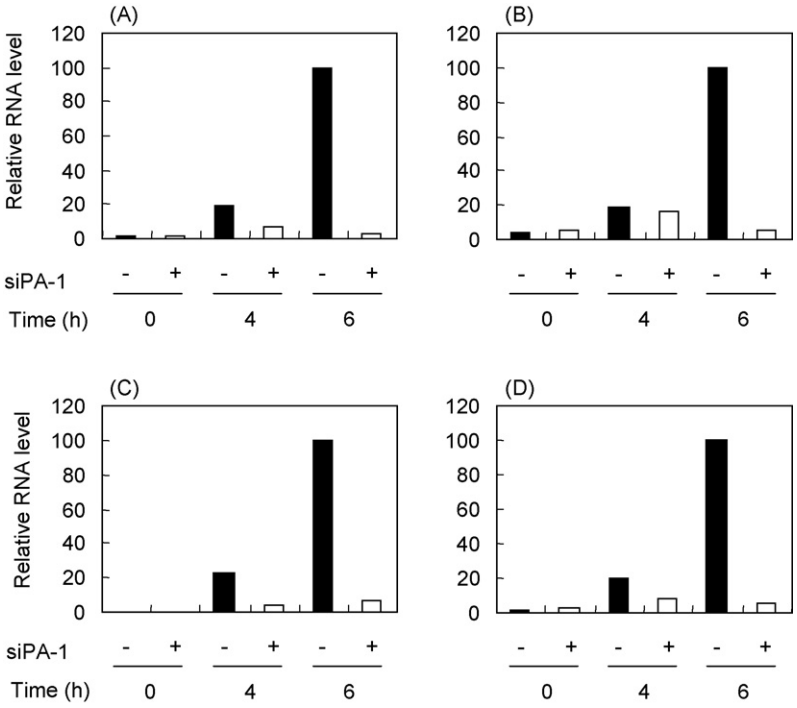


Fig. 2. Effects of PA-specific siRNA on accumulation of viral RNAs. MDCK cells were electroporated with 2.5 nmol/mL siPA-1, infected with PR8 virus at a MOI of 0.1, and harvested for RNA isolation 0, 4, and 6 h after infection. The levels of mRNA and vRNA specific to PA and NP were studied using RT with RNA-specific primers, followed by real-time PCR. The level of each viral RNA species is normalized to the level of β-actin mRNA in the same sample. The relative levels of RNAs are shown. (A) PA mRNA; (B) PA vRNA; (C) NP mRNA; (D) NP vRNA.

3.2. Intracellular inhibition of RNA polymerase by L-742,001

L-742,001 inhibited RNA polymerase activity and viral production (Fig. 1). We also investigated the effect of L-742,001 on the accumulation of viral RNA levels in infected cells. MDCK cells were infected with the PR8 virus at a MOI of 0.1 in the absence or presence of 10 μg/mL (=2 × IC_{50}) of L-742,001. At the indicated time after viral infection, PA mRNA, PA vRNA, NP mRNA, and NP vRNA in the infected cells were quanti-

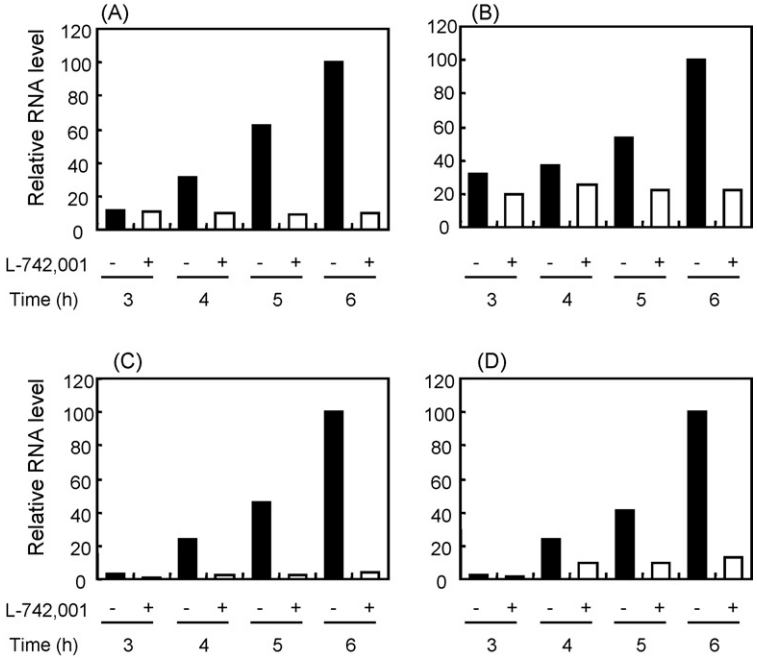


Fig. 3. Effect of L-742,001 on the accumulation of viral RNAs. MDCK cells were infected with the PR8 virus at a MOI of 0.1 in the presence of 10 μg/mL L-742,001 and harvested for RNA isolation at 3, 4, 5, and 6 h after infection. The levels of mRNA and vRNA specific to PA and NP were measured by RT using RNA-specific primers, followed by real-time PCR. The level of each viral RNA species is normalized to the level of 18S rRNA in the same sample. The relative levels of RNAs are shown. (A) PA mRNA; (B) PA vRNA; (C) NP mRNA; (D) NP vRNA.

Table 1
Isolation and characterization of L-742,001-resistant viruses

Virus	Passage no.	Drug conc. used for selection (μg/mL)	IC_{50} (μg/mL)[a]	PA82 cDNA sequence	PA20 amino acid residue
A/PR/8/34 (parent)	0	0	4.86	ACA	Thr
PC-2	P2	0	4.53	ND[b]	ND[b]
PC-6	P6	0	3.09	ACA	Thr
PL-1	P1	2	3.08	ACA	Thr
PL-2	P2	4	13.0	GCA	Ala
PL-6	P6	32	11.9	GCA	Ala

[a] Drug concentration required to reduce the number of plaques to 50% that of controls.
[b] Not determined.

fied by real-time PCR. In the absence of L-742,001, PA mRNA and PA vRNA increased time-dependently after the infection (Fig. 3A and B). In contrast, in the presence of L-742,001, PA mRNA and PA vRNA remained at base line levels. The same phenomenon was observed in the cases of NP mRNA and NP vRNA (Fig. 3C and D).

3.3. Characterization of L-742,001- and zanamivir-resistant viruses

The susceptibility of PL strains to L-742,001 was determined by plaque assay. PL-1 strain was as sensitive as the parent virus, whereas the PL-2 strain displayed a threefold lower susceptibility (Table 1). Also, the susceptibility of PL-6 strain to L-742,001 was almost the same as that of PL-2 strain (Table 1). When MDCK cells were infected with the resistant strain at a MOI of 0.001 in the presence of L-742,001 at 16 μg/mL, the resistant strain showed logarithmic growth. On the other hand, no growth was observed in the control strain at the same concentration (data not shown). These results indicate that PL strains except for the PL-1 strain show resistance to L-742,001.

To determine the amino acid changes in L-742,001-passaged strains, the cDNA sequences of RNA polymerase genes in PL-1, PL-2, and PL-6 strains were determined and compared with that of the parent virus. In L-742,001-resistant PL-2 and PL-6 strains, a single point mutation was observed at amino acid residue position 20 in the PA subunit of the cDNA (Thr-20 to Ala). On the other hand, no mutation was observed in PL-1 strain or PC-6 strain, which were sensitive to L-742,001. These results suggest that the amino acid residue position 20 in the PA subunit is correlated with susceptibility to L-742,001. As for PB1 and PB2 subunits, no mutation was observed in the amino acid sequences in the PL-2 strain. We also analyzed the cDNA sequences of NP, the other component of RNA polymerase complex involved in transcription/replication. However, no mutation was observed in NP of cDNA in the PL-2 strain.

To evaluate the sensitivity of the mutated enzyme to L-742,001, an RNA polymerase inhibitory assay was performed as described in Section 2. The PA-mutated enzyme showed two- to three-fold lower susceptibility to L-742,001 as compared with wild-type enzyme at the concentrations ranging from 0.37 μg/mL to 10 μg/mL. The IC_{50}s of L-742,001 against PA-mutated enzyme and wild-type enzyme in RNA polymerase assay were 1.24 μg/mL and 0.69 μg/mL, respectively.

Three zanamivir-resistant PZ-4 strains (PZ-4-1, PZ-4-2, and PZ-4-3) displayed high resistance (>30,000-fold) to zanamivir as compared with the parent strain (Table 2). A point mutation at the deduced amino acid residue position 119 in NA of cDNA (Glu to Gly or Asp; N2 numbering) was observed in these PZ-4 strains. These mutations correspond to the amino acid mutation observed previously in the zanamivir-resistant strains

Table 2
Antiviral activities of L-742,001 and zanamivir against L-742,001- and zanamivir-resistant viruses

Virus	Mutation			L-742,001 IC_{50} (μg/mL)[a]	Zanamivir IC_{50} (μg/mL)[a]
	PA	NA[b]	HA[c]		
Parent	Thr20	ND[d]	ND[d]	4.28	0.0188
PL-2	Thr20Ala	ND[d]	ND[d]	15.8	0.0208
Parent	ND[d]	Glu119	Wild-type	2.20	0.0289
PZ-4-1	ND[d]	Glu119Gly	Lys165Glu Leu320Pro	1.97	>1000
PZ-4-2	ND[d]	Glu119Gly	Lys144Glu	1.99	>1000
PZ-4-3	ND[d]	Glu119Asp	Lys144Glu	1.83	>1000

[a] Drug concentration required to reduce the number of plaques to 50% that of controls.
[b] Based on N2 numbering system (Colman et al., 1993).
[c] Based on H3 numbering system (Nobusawa et al., 1991).
[d] Not determined.

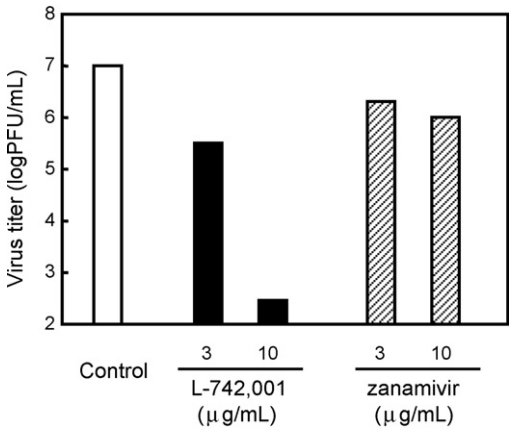


Fig. 4. Viral re-growth after the removal of L-742,001 or zanamivir. MDCK cells were infected with the PR8 virus at a MOI of 0.001. The cultures were treated with L-742,001 or zanamivir for 10 h and then the compounds were removed. Viral yields in the supernatant at 20 h post-infection were determined by a plaque assay.

(Blick et al., 1995; Staschke et al., 1995; Gubareva et al., 1997). All these strains also had one or two mutations in HA, especially Lys144Glu. These mutations in HA might contribute to the zanamivir resistance.

Although L-742,001 showed slight decreased activity against L-742,001-resistant strains, this compound was as effective against zanamivir-resistant strains as against the parent virus (Table 2).

3.4. Virus re-growth after exposure to L-742,001

To characterize the antiviral action of L-742,001, we examined the influenza virus inhibitory effect after removal of L-742,001 (Fig. 4). After influenza virus-infected MDCK cells were exposed to L-742,001 or zanamivir for 10 h, these agents were removed and the infected cells were continuously cultured for 10 h without the agents.

During the exposure to the antiviral agents, virus yields in the culture were below the detection limit. At 10 h after removal of zanamivir (20 h post-infection) at a dose of 10 μg/mL, that is 320 times the IC_{50} (0.0314 μg/mL), virus yield was increased to approximately the same level as the control. In contrast, at 10 h after removal of L-742,001 at a dose of 10 μg/mL [2 times the IC_{50} (4.86 μg/mL)], viral yield in the culture remained at a low level around the detection limit. These results indicate that L-742,001 had a greater inhibitory effect on virus re-growth than zanamivir.

4. Discussion

The aim of this study was to evaluate the potential of RNA polymerase as a target of anti-influenza virus agents by employing RNAi technique and RNA polymerase inhibitor. To use L-742,001 as a representative compound of RNA polymerase inhibitors, we confirmed that the antiviral effect of L-742,001 was due to its RNA polymerase inhibitory activity.

When the infected MDCK cells were pre-treated with PA-specific siRNA, virus production was completely inhibited. siRNA-induced degradation of its specific mRNA (PA mRNA) and suppressed NP mRNA, NP vRNA, and PA vRNA. Similarly, the amounts of PA mRNA and NP mRNA were down-regulated when cells were treated with L-742,001. Moreover, L-742,001 inhibited the accumulation of PA vRNA and NP vRNA. It was reported that diketobutanoic acids, including L-742,001, did not inhibit elongation but inhibited the endonucleolytic processing of RNA for transcription (Tomassini et al., 1994). The inhibitory effect of L-742,001 on mRNA accumulation is due to its endonuclease inhibitory activity, whereas the down-regulation of vRNA may be due to the decrease of de novo synthesis of RNA polymerase.

L-742,001 and PA-specific siRNA greatly prevented viral transcription and replication, leading to the inhibition of viral production. These results suggest that RNA polymerase is essential for all viral transcription and replication. In contrast, Ge et al. (2003) reported that M-specific siRNA inhibited only M mRNA and not M vRNA or other viral RNAs, since M-protein is not required until a late stage of virus infection. As Ge et al. discussed, these results reflect the difference of mode of action between RNA polymerase inhibitors and M protein inhibitors.

To study the specific target of L-742,001 genetically, L-742,001-resistant viruses were generated. We have detected a single mutation at the amino acid residue position 20 in the PA subunit of L-742,001-resistant viruses. These viruses exhibited approximately threefold lower susceptibility to L-742,001. A reduction in the susceptibility to L-742,001 was also observed at the enzyme level.

There is substantial variation at position 20 in PA among various influenza A viruses. So we analyzed PA gene sequence in several influenza A viruses and studied their susceptibility to L-742,001. A/Panama/2007/99 and A/New Caledonia/20/99, which carried Ala at position 20 in PA, were less sensitive than A/PR/8/34 strain (IC_{50} for each virus was 22.2 μg/mL and 9.29 μg/mL, respectively, while IC_{50} for A/PR/8/34 was 1.95 μg/mL). On the other hand, A/Yamagata/120/86, which carried Thr at position 20 in PA, was sensitive (IC_{50} was 0.989 μg/mL). These results suggest that substitution to Ala at position 20 in the PA subunit is involved in L-742,001-resistance, and that L-742,001 targets the PA subunit of RNA polymerase, thus leading to the antiviral effect.

Despite the serial passages of L-742,001-resistant viruses, they showed low level resistance to L-742,001. On the other hand, the zanamivir-resistant viruses showed at least 30,000-fold resistance to zanamivir. It was also reported that the zanamivir- and oseltamivir-resistant viruses have up to 100,000-fold resistance to NA inhibitors (Gubareva et al., 1996; Tai et al., 1998). These data indicated that L-742,001 showed less decrease in activity against L-742,001-resistant viruses than NA inhibitors against NA inhibitor-resistant viruses. We should note, however, that NA inhibitor-resistant viruses often had some mutations in HA, which might influence the HA binding efficiency and contribute to NA inhibitor resistance. It is likely that the resistance observed in cell culture-based assay might be higher

than the resistance observed in vivo experiments. HA mutations were also observed in PZ-4 strains, suggesting that it might be correlated to the resistance against zanamivir. On the other hand, several researchers reported that NA inhibitor-resistant mutants showed 40–250-fold resistant to NA inhibitors as compared with the parent strains by NA inhibition assay (Blick et al., 1995; Staschke et al., 1995; Gubareva et al., 2001). At least, this information suggests that the resistant level of NA inhibitor-resistant virus is much higher than that of L-742,001-resistant virus.

L-742,001 strongly inhibited virus re-growth after removal of the agent. Another RNA polymerase inhibitor, T-705 (Furuta et al., 2005), also has been shown to inhibit re-growth of influenza virus (Furuta et al., 2002; Takahashi et al., 2003). On the other hand, rapid virus re-growth was observed after the removal of zanamivir. In the case of antibacterial agents, the inhibition of the re-growth of bacteria after removal of the drug from the culture is designated as post-antibiotic effect ("PAE"). PAE as well as pharmacokinetics is known to be an important factor for the determination of a clinical regimen (Craig, 1993). In antiviral agents, re-growth inhibition may also be important factor in the clinical outcome. The RNA polymerase inhibitor showed strong re-growth inhibition, therefore, this profile of RNA polymerase inhibitors is favorable, and different from NA inhibitors. L-742,001 might not be a candidate as anti-influenza virus agent in clinical settings, but a promising lead compound for future medicinal chemistry.

The influenza virus RNA polymerase, which has multifunctional activities, is a heterotrimer composed of the PB1, PB2, and PA subunits. The PB1 subunit plays a central role in the catalytic activities of the RNA polymerase (Li et al., 2001). The PB2 subunit binds to cap structures of host pre-mRNA molecules (Ulmanen et al., 1981). Early studies suggested that PB2 was also responsible for endonuclease activity (Shi et al., 1995; Blok et al., 1996), but it was recently reported that the endonuclease active site is located in PB1 (Li et al., 2001). Since L-742,001 inhibits endonuclease activity of RNA polymerase (Hastings et al., 1996), we expected that the amino acid change of L-742,001-resistant viruses would have occurred in PB1 or PB2 subunits. Unexpectedly, it was detected in the PA subunit. The role of the PA subunit is not well established. It has been suggested that PA is associated with RNA replication (Perales et al., 2000; Honda et al., 2002). However, recent studies indicated that PA is involved in transcription as well as replication (Fodor et al., 2002; Regan et al., 2006). Recently, it was reported that the N-terminal region of PA plays a critical role in multiple functions, such as cap-binding, endonuclease activity, etc. (Hara et al., 2006). Since the L-742,001-resistant mutants have a mutation in N-terminal region of PA subunit, our results support that PA is involved in not only replication but also transcription. A three-dimensional structural model of a recombinant influenza virus RNA polymerase was reported, and it was suggested that there were extensive contacts among the three polymerase subunits (Area et al., 2004). Since L-742,001 is most likely to act at the PA subunit, the investigation with L-742,001 would improve the precision of the molecular modeling of RNA polymerase, and enhance the design of RNA polymerase inhibitors for anti-influenza virus agents.

In this paper, we evaluated the possibility of RNA polymerase as a target of anti-influenza virus agents. We demonstrated that RNA polymerase inhibitors possess mechanisms different from those of existing anti-influenza virus agents and perform interesting functions such as an inhibitory effect on re-growth. It is also suggested that the PA subunit of RNA polymerase can be a possible target of anti-influenza virus agents. Further study of the RNA polymerase, including the PA subunit, will promote the development of new agents that are valuable for the treatment of influenza virus infections in clinical use.

References

Area, E., Martín-Benito, J., Gastaminza, P., Torreira, E., Valpuesta, J.M., Carrascosa, J.L., Ortín, J., 2004. 3D structure of the influenza virus polymerase complex: localization of subunit domains. Proc. Natl. Acad. Sci. U.S.A. 101, 308–313.

Blick, T.J., Tiong, T., Sahasrabudhe, A., Varghese, J.N., Colman, P.M., Hart, G.J., Bethell, R.C., McKimm-Breschkin, J.L., 1995. Generation and characterization of an influenza virus neuraminidase variant with decreased sensitivity to the neuraminidase-specific inhibitor 4-guanidino-Neu5Ac2en. Virology 214, 475–484.

Blok, V., Cianci, C., Tibbles, K.W., Inglis, S.C., Krystal, M., Digard, P., 1996. Inhibition of the influenza virus RNA-dependent RNA polymerase by antisera directed against the carboxy-terminal region of the PB2 subunit. J. Gen. Virol. 77, 1025–1033.

Bouloy, M., Plotch, S.J., Krug, R.M., 1978. Globin mRNAs are primers for the transcription of influenza viral RNA in vitro. Proc. Natl. Acad. Sci. U.S.A. 75, 4886–4890.

Carr, J., Ives, J., Kelly, L., Lambkin, R., Oxford, J., Mendel, D., Tai, L., Roberts, N., 2002. Influenza virus carrying neuraminidase with reduced sensitivity to oseltamivir carboxylate has altered properties in vitro and is compromised for infectivity and replicative ability in vivo. Antiviral Res. 54, 79–88.

Cheer, S.M., Wagstaff, A.J., 2002. Spotlight on zanamivir in influenza. Am. J. Respir. Med. 1, 147–152.

Cianci, C., Chung, T.D.Y., Meanwell, N., Putz, H., Hagen, M., Colonno, R.J., Krystal, M., 1996. Identification of *N*-hydroxamic acid and *N*-hydroxyimide compounds that inhibit the influenza virus polymerase. Antiviral Chem. Chemother. 7, 353–360.

Colman, P.M., Hoyne, P.A., Lawrence, M.C., 1993. Sequence and structure alignment of paramyxovirus hemagglutinin-neuraminidase with influenza virus neuraminidase. J. Virol. 67, 2972–2980.

Craig, W.A., 1993. Post-antibiotic effects in experimental infection models: relationship to in-vitro phenomena and to treatment of infections in man. J. Antimicrob. Chemother. 31 (Suppl. D), 149–158.

Fodor, E., Crow, M., Mingay, L.J., Deng, T., Sharps, J., Fechter, P., Brownlee, G.G., 2002. A single amino acid mutation in the PA subunit of the influenza virus RNA polymerase inhibits endonucleolytic cleavage of capped RNAs. J. Virol. 76, 8989–9001.

Fukuda, K., Bridges, C.B., Brammer, T.L., Izurieta, H.S., Cox, N.J., 1999. Prevention and control of influenza: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR 48, 1–28.

Furuta, Y., Takahashi, K., Fukuda, Y., Kuno, M., Kamiyama, T., Kozaki, K., Nomura, N., Egawa, H., Minami, S., Watanabe, Y., Narita, H., Shiraki, K., 2002. In vitro and in vivo activities of anti-influenza virus compound T-705. Antimicrob. Agents Chemother. 46, 977–981.

Furuta, Y., Takahashi, K., Kuno-Maekawa, M., Sangawa, H., Uehara, S., Kozaki, K., Nomura, N., Egawa, H., Shiraki, K., 2005. Mechanism of action of T-705 against influenza virus. Antimicrob. Agents Chemother. 49, 981–986.

Ge, Q., McManus, M.T., Nguyen, T., Shen, C.H., Sharp, P.A., Eisen, H.N., Chen, J., 2003. RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. Proc. Natl. Acad. Sci. U.S.A. 100, 2718–2723.

Ge, Q., Filip, L., Bai, A., Nguyen, T., Eisen, H.N., Chen, J., 2004. Inhibition of influenza virus production in virus-infected mice by RNA interference. Proc. Natl. Acad. Sci. U.S.A. 101, 8676–8681.

Gubareva, L.V., Bethell, R., Hart, G.J., Murti, K.G., Penn, C.R., Webster, R.G., 1996. Characterization of mutants of influenza A virus selected with the neuraminidase inhibitor 4-guanidino-Neu5Ac2en. J. Virol. 70, 1818–1827.

Gubareva, L.V., Robinson, M.J., Bethell, R.C., Webster, R.G., 1997. Catalytic and framework mutations in the neuraminidase active site of influenza viruses that are resistant to 4-guanidino-Neu5Ac2en. J. Virol. 71, 3385–3390.

Gubareva, L.V., Webster, R.G., Hayden, F.G., 2001. Comparison of the activities of zanamivir, oseltamivir, and RWJ-270201 against clinical isolates of influenza virus and neuraminidase inhibitor-resistant variants. Antimicrob. Agents Chemother. 45, 3403–3408.

Hara, K., Schmidt, F.I., Crow, M., Brownlee, G.G., 2006. Amino acid residues in the N-terminal region of the PA subunit of influenza A virus RNA polymerase play a critical role in protein stability, endonuclease activity, cap binding, and virion RNA promoter binding. J. Virol. 80, 7789–7798.

Hastings, J.C., Selnick, H., Wolanski, B., Tomassini, J.E., 1996. Anti-influenza virus activities of 4-substituted 2,4-dioxobutanoic acid inhibitors. Antimicrob. Agents Chemother. 40, 1304–1307.

Hayden, F.G., Couch, R.B., 1992. Clinical and epidemiological importance of influenza A viruses resistant to amantadine and rimantadine. Med. Virol. 2, 89–96.

Herlocher, M.L., Carr, J., Ives, J., Elias, S., Truscon, R., Roberts, N., Monto, A.S., 2002. Influenza virus carrying an R292K mutation in the neuraminidase gene is not transmitted in ferrets. Antiviral Res. 54, 99–111.

Herlocher, M.L., Truscon, R., Elias, S., Yen, H.L., Roberts, N.A., Ohmit, S.E., Monto, A.S., 2004. Influenza viruses resistant to the antiviral drug oseltamivir: transmission studies in ferrets. J. Infect. Dis. 190, 1627–1630.

Honda, A., Mizumoto, K., Ishihama, A., 2002. Minimum molecular architectures for transcription and replication of the influenza virus. Proc. Natl. Acad. Sci. U.S.A. 99, 13166–13171.

Kaiser, L., Wat, C., Mills, T., Mahoney, P., Ward, P., Hayden, F., 2003. Impact of oseltamivir treatment on influenza-related lower respiratory tract complications and hospitalizations. Arch. Int. Med. 163, 1667–1672.

Kiso, M., Mitamura, K., Sakai-Tagawa, Y., Shiraishi, K., Kawakami, C., Kimura, K., Hayden, F.G., Sugaya, N., Kawaoka, Y., 2004. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. Lancet 364, 759–765.

Le, Q.M., Kiso, M., Someya, K., Sakai, Y.T., Nguyen, T.H., Nguyen, K.H., Pham, N.D., Ngyen, H.H., Yamada, S., Muramoto, Y., Horimoto, T., Takada, A., Goto, H., Suzuki, T., Suzuki, Y., Kawaoka, Y., 2005. Avian flu: isolation of drug-resistant H5N1 virus. Nature 437, 1108.

Li, M.L., Rao, P., Krug, R.M., 2001. The active sites of the influenza cap-dependent endonuclease are on different polymerase subunits. EMBO J. 20, 2078–2086.

Nichol, K.L., Wuorenma, J., von Sternberg, T., 1998. Benefits of influenza vaccination for low-, intermediate-, and high-risk senior citizens. Arch. Int. Med. 158, 1769–1776.

Nobusawa, E., Aoyama, T., Kato, H., Suzuki, Y., Tateno, Y., Nakajima, K., 1991. Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses. Virology 182, 475–485.

Parkes, K.E., Ermert, P., Fässler, J., Ives, J., Martin, J.A., Merrett, J.H., Obrecht, D., Williams, G., Klumpp, K., 2003. Use of a pharmacophore model to discover a new class of influenza endonuclease inhibitors. J. Med. Chem. 46, 1153–1164.

Perales, B., Sanz-Ezquerro, J.J., Gastaminza, P., Ortega, J., Santarén, J.F., Ortín, J., Nieto, A., 2000. The replication activity of influenza virus polymerase is linked to the capacity of the PA subunit to induce proteolysis. J. Virol. 74, 1307–1312.

Regan, J.F., Liang, Y., Parslow, T.G., 2006. Defective assembly of influenza A virus due to a mutation in the polymerase subunit PA. J. Virol. 80, 252–261.

Shi, L., Summers, D.F., Peng, Q., Galarz, J.M., 1995. Influenza A virus RNA polymerase subunit PB2 is the endonuclease which cleaves host cell mRNA and functions only as the trimeric enzyme. Virology 208, 38–47.

Staschke, K.A., Colacino, J.M., Baxter, A.J., Air, G.M., Bansal, A., Hornback, W.J., Munroe, J.E., Laver, W.G., 1995. Molecular basis for the resistance of influenza viruses to 4-guanidino-Neu5Ac2en. Virology 214, 642–646.

Tai, C.Y., Escarpe, P.A., Sidwell, R.W., Williams, M.A., Lew, W., Wu, H., Kim, C.U., Mendel, D.B., 1998. Characterization of human influenza virus variants selected in vitro in the presence of the neuraminidase inhibitor GS 4071. Antimicrob. Agents Chemother. 42, 3234–3241.

Takahashi, K., Furuta, Y., Fukuda, Y., Kuno, M., Kamiyama, T., Kozaki, K., Nomura, N., Egawa, H., Minami, S., Shiraki, K., 2003. In vitro and in vivo activities of T-705 and oseltamivir against influenza virus. Antiviral Chem. Chemother. 14, 235–241.

Tomassini, J., Selnick, H., Davies, M.E., Armstrong, M.E., Baldwin, J., Bourgeois, M., Hastings, J., Hazuda, D., Lewis, J., McClements, W., et al., 1994. Inhibition of cap (m7GpppXm)-dependent endonuclease of influenza virus by 4-substituted 2,4-dioxobutanoic acid compounds. Antimicrob. Agents Chemother. 38, 2827–2837.

Tomassini, J.E., Davies, M.E., Hastings, J.C., Lingham, R., Mojena, M., Raghoobar, S.L., Singh, S.B., Tkacz, J.S., Goetz, M.A., 1996. A novel antiviral agent which inhibits the endonuclease of influenza viruses. Antimicrob. Agents Chemother. 40, 1189–1193.

Tompkins, S.M., Lo, C.Y., Tumpey, T.M., Epstein, S.L., 2004. Protection against lethal influenza virus challenge by RNA interference in vivo. Proc. Natl. Acad. Sci. U.S.A. 101, 8682–8686.

Ulmanen, I., Broni, B.A., Krug, R.M., 1981. Role of two of the influenza virus core P proteins in recognizing cap 1 structures (m7GpppNm) on RNAs and in initiating viral RNA transcription. Proc. Natl. Acad. Sci. U.S.A. 78, 7355–7359.

World Health Organization, 2004. Influenza: Report by the Secretariat. World Health Assembly, WHO, Geneva, A56/23.

Yamashita, M., Krystal, M., Palese, P., 1989. Comparison of the three large polymerase proteins of influenza A, B, and C viruses. Virology 171, 458–466.