口蹄疫ウイルス RNA 依存性 RNA ポリメラーゼに対する抗ウイルス剤のインビトロ活性

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In vitro Activities of Antiviral Agents Against Foot-and-Mouth Disease Virus RNA-dependent RNA Polymerase

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Summary

Through a comparison of amino acid sequences between foot-and-mouth disease virus (FMDV) 3D and poliovirus 3D proteins whose three-dimensional structure had already been determined by X-ray analysis, the structure of the FMDV 3D protein was predicted by three-dimensional construction software. Since the structures of the RNA and DNA polymerases (DNA-dependent DNA polymerase, DNA-dependent RNA polymerase, RNA-dependent DNA polymerase (reverse transcriptase) and RNA-dependent RNA polymerase) are homeomorphous, antiviral efficacies against FMDV were found in the Non-Nucleotide Reverse Transcriptase Inhibitors of the anti-Human Immunodeficiency Virus (HIV), and in pyrazinecarboxamide derivatives which were considered to be RNA polymerase inhibitors. The inhibition concentration (IC_{50}) of Efavirenz, one of the Non-Nucleotide Reverse Transcriptase Inhibitors, was between 20-40μg/ml. Meanwhile, a pyrazinecarboxamide derivative, T-1105, showed the strongest efficacy of the inhibitors, and its IC_{50} was 1.6μg/ml according to 50% plaque reduction assays. T-1105 was also effective for other serotypes (type A, C, Asian 1) of FMDV.

Keywords : antiviral agents, foot-and-mouth disease, anti-HIV agents, pyrazinecarboxamide derivatives, RNA-dependent RNA polymerase, 3D protein

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Introduction

Foot-and-mouth disease (FMD) is a highly contagious and economically devastating disease of cloven-hoofed animals[1]. The causative agent, foot-and-mouth disease virus (FMDV), is an *aphthovirus* of the *picornaviridae* family and is serologically classified into seven distinct serotypes such as O, A, C, South African Territories (SAT) 1, SAT 2, SAT 3 and Asia 1[1,9]. The diseases caused by those different serotypes are clinically indistinguishable[7].

The economic impact of FMD can be catastrophic, when the outbreak occurs in FMD free countries where naïve animals are not vaccinated. The pig plays a role as an amplifier in the outbreaks because they excrete the viruses 1,000 – 2,000 times more than other susceptible animals do[4]. FMD virus particles are transported by the wind and it will often be a new origin of infection and the disease spreads rapidly[4]. Examples of such outbreaks occurred in 1997 in Taiwan[6] and in 2001 in the UK[10]. Those findings suggest that controlling FMD in pigs is one of the most important factors in avoiding huge expansions of FMD outbreaks.

The main policies to control FMD are "test and slaughter" and/or "vaccination". Countries where FMD outbreaks occur will decide to use either or both approaches,

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depending on the epidemiological situation. Since FMDV-infected animals excrete the virus before they produce effective antibodies to protect themselves from FMDV infection, FMDV transmits rapidly in susceptible animals. The rate of transmission depends on a multitude of factors among which are the infected species and breed, the FMDV strain involved, the virulence of the virus strain, its infection route, the environment and differences in the immune status of the individual animal and the quality of the FMD vaccines; however, generally it takes about 7 days to produce effective protective antibodies to FMD after vaccination[3]. To compensate for this disadvantage of FMD vaccines, new control methods which exhibit prompt effectiveness is required.

Several *in vitro* studies on the antiviral agents against FMDV have been reported[2, 11, 12, 16, 18]. In the present study we estimated the structure of the FMDV 3D protein (RNA-dependent RNA polymerase)[5] with computer software by comparing its amino acid sequence with that of poliovirus 3D protein, and several anti-FMDV activities of anti-RNA polymerase agents were evaluated by *in vitro* assay.

Materials and methods

1. Construction of the three-dimensional structure of the RNA-dependent RNA polymerase of FMDV

The structure of the FMDV 3D protein was estimated by using the three-dimensional construction software ICM version 3 (Molsoft, CA, USA) comparing with the amino acid sequence of the RNA-dependent RNA polymerase of poliovirus.

2. Viruses and cell

FMDV O/JPN/2000 was isolated from Japanese Black cattle in the FMD outbreak in Japan[20]. It was propagated with IBRS-2 cells, which were grown in Eagle's minimal essential medium containing 10% tryptose phosephate broth and 5% fatal calf serum. FMDV A22 Iraq, C Philippine and Asia 1 Shamir were obtained from the Institute for Animal Health in United Kingdom, one of the FMD reference laboratories of the World Organization for Animal Health (OIE).

3. Test materials

The three Non-Nucleotide Reverse Transcriptase Inhibitors of Anti-HIV agents, such as Nevirapine, Efavirenz and Delavirdine were commercially purchased. The other three pyrazinecarboxamide derivatives such as T-705, T-1105 and T-1106 were synthesized and supplied by Toyama Chemical Co., Ltd.

4. In vitro antiviral testing

The anti-FMDV activities of the compounds were investigated by means of 50% plaque reduction assays with monolayers of IBRS-2 cells in 6-well microplates infected with about 30-50 plaque forming units of FMDV, O/JPN/2000 in 200μl of MEM at 37℃ for 1 hr. The compounds were diluted in a series of 4-time concentrations in the overlayer of 1.5% methyl cellulose-MEM containing 2% newborn calf serum and 10% tryptose phosphate broth. The 50% inhibition concentration (IC_{50}) of the plaque formations were calculated by using the plaque counts of cultures with or without the compounds.

The antiviral activity of T-1105 against three other serotypes of FMDV, namely type A22 Iraq, type C Philippine and type Asia 1 Shamir, was also examined by measuring the 50% CPE inhibition concentration with low titres of virus ($10^{1.0}$ – $10^{1.5}$ 50% tissue culture infectious dose ($TCID_{50}$)) in liquid phase.

Results

1. Estimated three-dimensional structure of RNA-dependent RNA polymerase of FMDV

Since the three-dimensional structure of the RNA-dependent RNA polymerase of poliovirus, which belongs to the same family as FMDV, had already been determined by X-ray analysis[13], the structure of the FMDV 3D protein was estimated using the three-dimensional construction software ICM version 3 (Molsoft, CA, USA). Although the homologies of the nucleotides and the amino acids between FMDV 3D[15] and that of poliovirus were less than 50% and approximately 30%, respectively, this software could construct the structure of FMDV RNA polymerase. The structure of FMDV RNA polymerase had a so-called right-hand shape and was similar to the structures of other polymerases (Fig. 1)[21].

2. In vitro antiviral activities of the Non-Nucleotide Reverse Transcriptase Inhibitors and the pyrazinecarboxamide derivatives, T-705, T-1105 and T-1106, against FMDV O/JPN/2000

The IC_{50} of the Non-Nucleotide Reverse Transcriptase Inhibitors are shown in Table 1. In the Non-Nucleotide Reverse Transcriptase Inhibitors only Efavirenz possessed anti-FMDV activity and its IC_{50} was 20-40μg/ml. On the other hand, the antiviral activities of pyrazinecar-

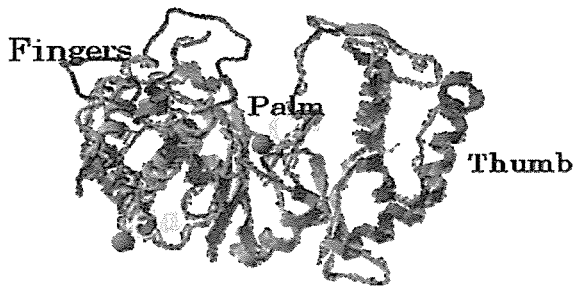

Fig. 1. The three-dimensional structure of FMDV RNA-dependent RNA polymerase was estimated using the three-dimensional construction software ICM version 3 (Molsoft, CA, USA) by comparing its amino acid sequence with that of poliovirus RNA polymerase.

Table 1. Anti-FMDV RNA-dependent RNA polymerase materials activity

Group of Compound	Name of Agent	IC_{50} (μg/ml)
Anti-HIV agents		
(Non-Nucleotide	Nevirapine	1000<
Reverse Transcriptase	Efavirenz	20－40
Inhibitors)	Delavirdine	1000<
Pyrazinecarboxamide	T-705	14
Derivatives	T-1105	1.6
	T-1106	17

Virus : FMDV O/JPN/2000 Cells : IBRS-2

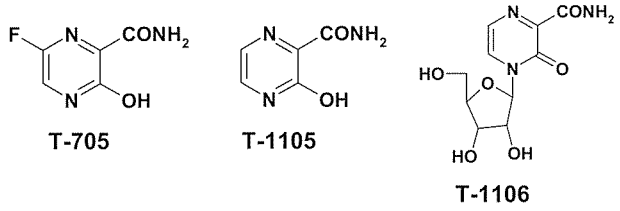

Fig. 2. Chemical formulas of the pyrazinecarboxamide derivatives, T-705, T-1105 and T1106

boxamide derivatives such as T-705, T-1105 and T-1106 (Chemical formura as shown in Fig. 2) were 1.6 – 17μg/ml. T-1105 of the derivatives has the strongest among them, and its IC_{50} was 1.6μg/ml. The cytotoxity of T-1105 was not observed at the highest dose (100μg/ml) in IBRS-2 cells.

Table 2. Effect of T-1105 on FMDV different serotype strains

FMDV strain	Test infectious dose	50% CPE Inhibition Concentration (μg/ml)
A 22 Iraq	$10TCID_{50}$	0.44
C Philippine	$10TCID_{50}$	1.76
Asia 1 Shamir	$10^{1.5}TCID_{50}$	0.88

3. In vitro antiviral activity of T-1105 against FMDV type A, C and Asia 1

When using FMDV type A, C and Asia 1, small pinpoint plaques were only observed even in the wells with a higher concentration of T-1105, and by this phenomenon it is not able to calculate the plaque numbers. From the reason the antiviral activities of T-1105 against three other serotypes of FMDV, namely type A, C and Asia 1, were examined by measuring the 50% CPE inhibition concentration with $10^{1.0}$ – $10^{1.5}$ 50% tissue culture infectious dose ($TCID_{50}$) viruses in liquid phase. The effectiveness of T-1105 against these three different serotypes of FMDV strains according to this method is shown in Table 2. T-1105 also showed good efficacy against these three serotypes, and their 50% cytopathic effect (CPE) inhibition doses were 0.44 to 1.76μg/ml.

Discussion

The structures of the three types of polymerases, namely DNA-dependent DNA polymerase, DNA-dependent RNA polymerase, and RNA-dependent DNA polymerase (reverse transcriptase) can also be described as analogous to a right hand, consisting of a palm, fingers, and thumb [17]. RNA-dependent RNA polymerase has the same overall shape as other polymerases, although the fingers and the thumb are different from those of other polymerases [13]. Since the palm domain contains the active site of the enzyme and the domain is similar to that of other polymerases, the anti-FMDV activities of the Non-Nucleotide Reverse Transcriptase Inhibitors of anti-Human Immunodificiency Virus (HIV) agents, Efavirenz, Nevirapine and Delavirdine could be considered to be good candidates of the antiviral agents against FMDV. However the antiviral activity of T-1105 to FMDV was approximately 10 times higher than those of the other pyrazinecarboxamide derivatives and 12.5-25 times higher than that of the anti-HIV agent, Efavirenz. The structure

of RNA-dependent RNA polymerase has the same overall shape with that of RNA-dependent DNA polymerase (reverse transcriptase). Since especially their palm domains which contain the enzyme active site, are similar each other, this can be the reason why an anti-HIV agent, Efavirenz possessed anti-FMDV activity. However its low activity may come from the different pocket sizes of the active sites of both enzymes.

On the other hand T-1105 possessed the enough anti-FMDV activities to all tested FMDV serotypes and it showed that the efficacy of T-1105 against FMDV did not depend on the FMDV serotypes. This finding represents an excellent advantage of this anti-FMDV agent comparing with FMD vaccine, when it is considered to be used in FMDV-infected pigs.

Further more the cytotoxity of T-1105 was not observed at the highest concentration of 100μg/ml in IBRS-2 cells. The pyrazinecarboxamide derivative T-705, a chemical analog of T-1105, showed no cytotoxicity at concentrations up to 1,000μg/ml, on the other hand, that of amantadine was 160μg/ml and ribavirin was 23μg/ml in MDBK cells[8]. It is considered that the derivatives have selective inhibition action and a wide margin of safety.

Based on this *in vitro* experiment, T-1105, which is considered as an RNA-dependent RNA polymerase inhibitor[8, 9, 14], can be a good candidate for reducing the excretion of FMDV by infected pigs. Appling to the *in vivo* research work with this compound, the pharmacokinetics of T-1105 in pigs must be known, and the maintenance of an effective concentration of T-1105 in the plasma will be especially critical after the administration of T-1105 to pigs.

RNA-dependent RNA polymerase is the essential enzyme for virus propagation. Since a prompt effect of the antiviral agent to inhibit FMDV infection or propagation can be expected, the strategy of using antiviral agents in FMD outbreaks may be another approach to controlling FMD. In the control of FMD in FMD free countries in which vaccination is not applied, the use of the antiviral agent could be considered as a powerful tactic to reduce the expansion of FMD infection. This approach to FMD control is significantly different from the vaccine approach.

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要　約

〈口蹄疫ウイルスRNA依存性RNAポリメラーゼに対する抗ウイルス剤のインビトロ活性〉

X線解析により既にその立体構造が明らかにされているポリオウイルスの3D蛋白質（RNA依存性RNAポリメラーゼ）のアミノ酸配列と口蹄疫ウイルスの3D蛋白質のアミノ酸配列を比較して、口蹄疫ウイルスのRNA依存性RNAポリメラーゼの3次元構造をコンピュータ解析により予測した。また、DNAおよびRNAポリメラーゼであるDNA依存性DNAポリメラーゼ、DNA依存性RNAポリメラーゼ、RNA依存性DNAポリメラーゼ（逆転写酵素）など他のポリメラーゼ群と、RNA依存性DNAポリメラーゼが類似した構造を有していることから、ヒト免疫不全ウイルス（HIV）のRNA依存性DNAポリメラーゼである逆転写酵素の阻害剤、非核酸系逆転写阻害剤の1種とRNAポリメラーゼ阻害剤と考えられるピラジンカルボキサミド誘導体に口蹄疫ウイルスにも増殖阻害効果があることを明らかにした。非核酸系逆転写阻害剤エファビレンツの50％阻止濃度が20－40μg/mlであったのに対して、ピラジンカルボキサミド誘導体の1種、T-1105に50％プラック減少法において1.6μg/mlの濃度で最も強い抗口蹄疫ウイルス活性があることを見出した。この活性は複数の口蹄疫ウイルス血清型に同程度の有効性を示したことから、T-1105は血清型に依存するワクチンとは異なり、ウイルスの排泄を抑制でき、口蹄疫の防圧に際して有用な資材となる可能性があると考えられる。

キーワード：抗ウイルス剤、口蹄疫、HIV阻害剤、ピラジンカルボキサミド誘導体、RNA依存性RNAポリメレース、3D蛋白質