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Original Article

Characterization of susceptibility variants of poliovirus grown in the presence of favipiravir

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Abstract *Background:* T-705 (favipiravir) is a potent inhibitor of RNA-dependent RNA polymerases of influenza viruses and no favipiravir-resistant virus has been isolated. Poliovirus RNA polymerase has been well characterized and isolation of resistant virus was examined in poliovirus.
Methods: Susceptibility variants of poliovirus I (Sabin strain) were isolated during passages in the presence of favipiravir and characterized for their susceptibility and the sequence of RNA polymerase.
Results: Five variants with 0.47—1.88 times the 50% inhibitory concentration for plaque formation of the parent poliovirus had amino acid variations in the 3D gene of the RNA polymerase. The distribution of amino acid variations was not related to ribavirin resistance, and two amino acid variation sites were found near the finger domain.
Conclusion: Favipiravir as a chain terminator would not be incorporated and replicate to cause lethal mutagenesis as a mutagen like ribavirin, and resistant mutants were not isolated. A high replication level would generate mutations leading to favipiravir resistance as ribavirin resistance was generated, but generated mutations would be lethal to the RNA polymerase function.
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Introduction

We have developed T-705 (favipiravir) as a viral RNA synthesis inhibitor and its anti-influenza activity and efficacy have been shown in influenza virus infection in animal models.[1–3] Favipiravir has been approved to treat novel or re-emerging influenza viruses in Japan. The Taiwanese CDC's decided to stockpile favipiravir for the people who become infected with a new strain of influenza including avian and swine influenzas.

Favipiravir has been shown to have a broad spectrum against RNA viruses in animal infection models[4,5]; Influenza virus infection,[1–5] Ebola virus (EBOV) infection,[6] Crimean-Congo hemorrhagic fever,[7] Lassa virus infection,[8] Rift Valley fever, Arenavirus hemorrhagic fever infections, Chikungunya virus infection,[9] Severe fever with thrombocytopenia syndrome virus infection,[10] Rabies,[11] Yellow fever virus disease, Hantavirus pulmonary syndrome,[12] Western equine encephalitis, West Nile virus infection, and Norovirus infection.[13] Based on the anti-EBOV efficacy in animal studies, favipiravir has been used for treatment of EBOV infection in Guinea and Sierra Leone and the promising results has been reported.[14,15]

Favipiravir inhibits viral RNA-dependent RNA polymerase replication by inhibiting elongation at the incorporation site as a chain terminator.[16,17] This mechanism does not produce mutations in the replicating genome, and accordingly we failed to isolate resistant mutants of influenza virus but did find variants with less susceptibility to favipiravir.[18] Poliovirus 3D RNA-dependent RNA polymerase has been well characterized in its structure, function and resistance to ribavirin.[19–23] We have tried to isolate favipiravir-resistant poliovirus to elucidate the interaction of favipiravir and viral RNA-dependent RNA polymerase in this study.

Methods

Virus, cells, and reagents

We used poliovirus (Sabin 1) for characterization of the susceptible variants and their genome sequences obtained in the presence of favipiravir[24] because it is a very frequently used laboratory strain with the best characterized sequence data on the genome containing naturally-occurring variants. Vero cells were grown and maintained in Eagle's minimum essential medium supplemented with 2% and 5% heat-inactivated calf serum, respectively.[24] Favipiravir was provided from Toyama Chemical Co., Ltd.

Isolation of susceptibility variants

Vero cells in 25 cm^2 flasks were infected with poliovirus, and 10 flasks were independently cultured in the presence of stepwise increasing concentrations of 10, 30, and 50 μg/ml of favipiravir for 3–7 days with a medium change depending on the spread of the cytopathic effect (CPE), rounding of the cells, to the whole cells. When the CPE developed in a flask containing 5 μg/ml of favipiravir, an aliquot of 200 μl of the culture supernatant after freezing and thawing the culture three times was inoculated into a new 25 cm^2 flask for further incubation in the presence of 10 μg/ml of favipiravir. Thus favipiravir concentration was increased at 5, 10, 30, and 50 μg/ml of favipiravir. When a culture showed extensive CPE in the whole culture in the presence of 50 μg/ml of favipiravir, the infected cells were frozen and thawed three times, and the supernatants were inoculated into Vero cells in 60-mm plastic flasks with 0.8% nutrient methylcellulose medium at 50 μg/ml of favipiravir. The plaques were isolated and the isolated virus was examined for the susceptibility to favipiravir.

Determination of susceptibility to favipiravir

Susceptibility to favipiravir was determined by a plaque reduction assay.[18,24] Duplicate cultures of Vero cells in 60-mm plastic dishes were infected with 100 plaque forming units (PFU)/0.2 ml of poliovirus or isolated clones for 1 h at room temperature. Cells were overlaid with 5 ml of 0.8% nutrient methylcellulose medium containing various concentrations of favipiravir and then cultured at 37 °C for 2–3 days. The infected cells were fixed with 5% formalin solution and stained with 0.03% methylene blue solution. The number of plaques was counted under a dissecting microscope. The inhibitory concentrations of favipiravir for 50% plaque reduction (IC_{50}) were determined from a curve relating the plaque number to the favipiravir concentration. The susceptibility of an isolated clone was expressed as the mean ratio of the IC_{50} of the isolated clone over that of the original poliovirus virus assayed simultaneously to minimize the variation of the IC_{50} values between the original and cloned viruses.

Determination of nucleotide sequences of susceptibility variants

The variations in the change of IC_{50} values were linked to amino acid changes in the 3D RNA polymerase gene and the nucleotide sequences of the RNA polymerase were determined in 7 clones and original virus. RNA extraction was demonstrated by using a QIAamp viral RNA mini kit (QIAGEN GmbH, Germany) and the viral RNA was reverse-transcribed with the Oligo (dT)15 Primer using the PrimeScript RT reagent Kit (Takara Bio, Otsu, Shiga). The amplified segments of the RNA polymerase by PCR were sequenced by using the ABI Prism 3130 DNA sequencer.

The binding of T-705RTP to the active site of the poliovirus 3D RNA polymerase

In order to gain insight into how favipiravir binds to the active site of the 3D RNA polymerase, we performed a docking simulation analysis of favipiravir-ribose-5'-triphosphate (T-705RTP) and poliovirus RNA polymerase (PDB code 3OLA) using Autodock Vina.[25]

Results

Isolation of the susceptibility variants

The IC_{50}s of 18 candidate clones were determined in parallel with the parent virus twice and ranged from 3.21 to

12.79 μg/ml, corresponding to 0.47 to 1.88 times that of the parent virus. Seven susceptibility variant clones among 18 variants with sequence analysis were selected and reduced to 5 clones by the overlap of the amino acid mutation (Table 1). Amino acid variations observed in the isolated clones were marked in blue (more sensitive to favipiravir: lower IC_{50}) or red (less susceptible to favipiravir: higher IC_{50}) in the RNA polymerase gene as shown in Fig. 1. Thus, favipiravir-susceptibility variants were isolated, but favipiravir-resistant clones with IC_{50} values 3–5 times higher were not isolated from virus populations in infected cultures treated continuously with favipiravir for a month. These variants had plaque sizes similar to those of the parent virus in the absence of favipiravir. The amino acid variations in the gene coding the 3D RNA polymerase were not clustered nor distributed in the functional domain changes and not related to alterations of susceptibility in the RNA-dependent RNA polymerase gene,[20,22] as illustrated in Fig. 1.

Amino acid variation and the binding of T-705RTP to the active site of the poliovirus 3D RNA polymerase

Amino acid substitutions of the susceptibility variants were indicated in the three-dimensional structure of the 3D RNA-dependent RNA polymerase[20] in Fig. 2, and the amino acid substitutions were not localized near the active sites. In order to consider the possibility that favipiravir binds to the active site of the RNA polymerase, we performed docking simulation analysis of favipiravir-ribose-5′-triphosphate (T-705RTP) and the poliovirus RNA polymerase (PDB code 3OLA) using Autodock Vina.[21] The simulation predicted potential hydrogen bonds between the amino group of favipiravir and the carbonyl oxygen of Ser173, between the hydroxyl group of the ribose and the side chain amino group of Lys61, and between the phosphate group and the amide group of Asp328 (Fig. 3). Thus, the simulation predicts that T-705RTP inhibits the RNA polymerase by binding to its active site.

Table 1 Susceptibility (IC_{50}) and amino acid mutation of poliovirus susceptibility variants.

Clones	KA4	KA8	KWC1	KWC7	KA5	KA7	SA0
IC_{50} (μg/ml)	9.43	10.7	3.6	3.21	7.94	12.79	9.1
Ratio to parent[a]	1.38	1.57	0.53	0.47	1.17	1.88	1.34
Amino acid mutation	V12A A122T	V12A A122T	N18S D99G	N18S K395E	N18S	N18S	K139R

[a] The ratio was expressed as IC_{50} value of the variant per IC_{50} of the parent virus.
Seven susceptibility variants (KA4, KA8, KWC1, KWC7, KA5, KA7, SA0) were independently isolated, and two variants had the same mutation (KA4 and KA8, KA5 and KA7).

Discussion

Sequence analysis of favipiravir-treated viruses increased nucleotide substitutions (transition) in the genome.[13,17,26] The susceptibility variant of enterovirus (3D S121N) about twice of IC_{50} value, chikungunya virus (nsP4 K291R),[9] and influenza virus[18] accumulated in the cultures in presence of favipiravir. Thus various nucleotide substitutions were observed in the viral genome cultured in the presence of favipiravir but no resistant virus has been isolated.

Susceptibilities of some poliovirus clones to antiviral drugs are different but within around three times the IC_{50} of the original clone; these clones are defined as susceptibility variants and not drug-resistant mutants. The susceptibility variants within around three times the IC_{50} of the original clone indicate that these might be generated independently of favipiravir treatment and selected as susceptibility variants because of the IC_{50} change and amino acid variation in the 3D polymerase gene. The susceptibility variants with amino acid alterations in the presence of favipiravir showed increased and decreased susceptibilities to favipiravir compared to the parent clone. It is difficult to predict the relationship between amino acid alteration and susceptibility change. However, we speculated as follows: The N18S alteration may be related to

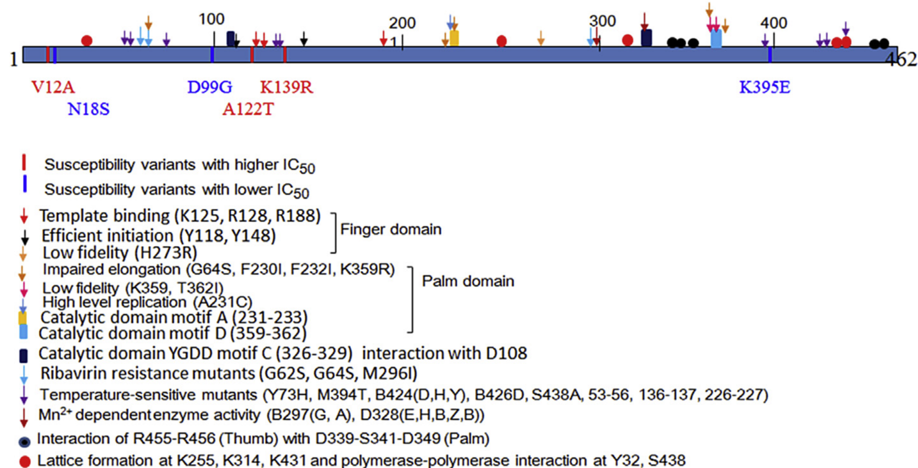

Figure 1. Locations of amino acid changes in the viral 3D RNA-dependent RNA polymerase gene of polioviruses generated during viral replication in the presence of favipiravir. The functional domains in the gene were modified from published illustrations. Fourteen amino acid variations of 6 susceptibility variants are indicated; variants with lower IC_{50} are marked in blue and those with higher IC_{50} are marked in red. Mutants listed are from.[19–23,27–30,32–40]

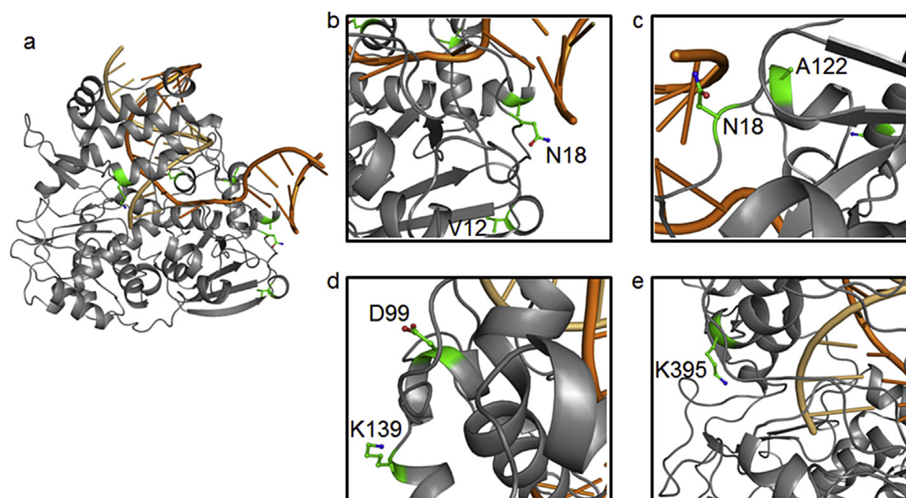

Figure 2. The positions of the amino acid substitutions in poliovirus RNA polymerase induced by favipiravir. (a) Structure of poliovirus RNA polymerase (PDB code 3OLA). Poliovirus RNA polymerase is grey. The substituted residues are green. (b—e) Expanded view of the substituted residues. The side chains are illustrated with ball-and-stick models.

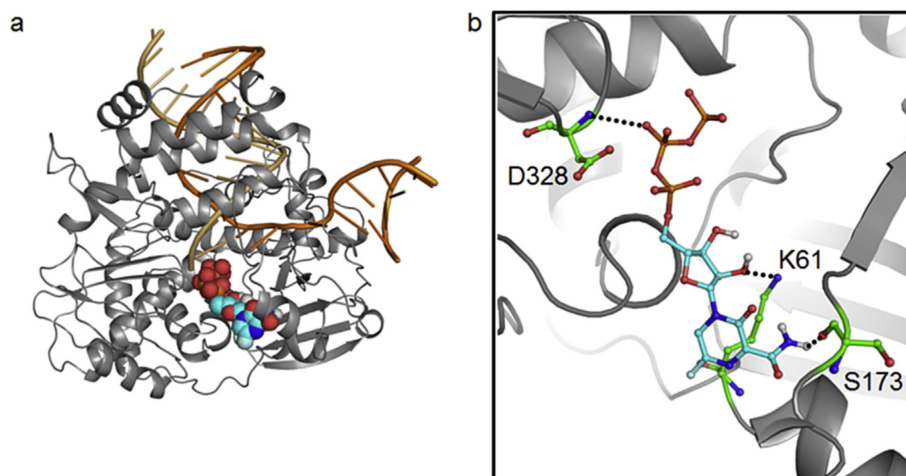

Figure 3. The binding of T-705RTP to the active site of poliovirus RNA polymerase. (a) Structure of the predicted complex between T-705RTP and poliovirus RNA polymerase (PDB code 3OLA). T-705RTP is represented as a sphere. Poliovirus RNA polymerase is grey. (b) T-705RTP is represented as a ball-and-stick model. The residues that are predicted to form hydrogen bonds with T-705RTP are also shown as ball-and-stick models. Hydrogen bonds are indicated by dotted lines.

interaction with nontemplate RNA. D99G may affect the α-helix structure. A122T and K139R were located near the template binding site of the finger domain[27] but apart from the fidelity-determining domain and ribavirin-recognizing domain,[23,28—30] indicating that favipiravir would be quite different from ribavirin. Amino acid changes of G62S, G64S, and M296I were important for recognition between ribavirin triphosphate (RTP) and adenosine/guanosine triphosphate and were not obtained in this study, suggesting the difference as the substrate for the RNA polymerase between RTP and favipiravir triphosphate. Ribavirin is incorporated into the replicating strand of the genome, and generates mismatched nucleotides as a template in the next complimentary strand formation, resulting in production of replication-competent mutants or lethal mutagenesis of the further replicating genome. Favipiravir stops elongation by a single incorporation of favipiravir[16] and two consecutive incorporation of favipiravir[17] and does not generate a mismatch of the nucleotides in the replicating genome, and further the RNA-dependent RNA polymerase has no proofreading activity,[31] indicating no chance that favipiravir will make a mismatch.

Spontaneous mutation rates of the poliovirus 3D RNA polymerase ranged from 1.3×10^{-5} to 2.3×10^{-3},[19,31] from 1.2×10^{-4} to 1.0×10^{-6} for transition mutations, and from 3.2×10^{-5} to 4.3×10^{-7} for transversion mutations or 1/20,000[30]. The poliovirus genome has about 7500 bases, indicating one spontaneous nucleotide alteration per genome might be generated during one cycle of replication. Virus titer in the culture reaches around 10^8/ml $\times$ 5 ml in T-25 flask and continued cultivation for a month. This indicated as follows. At least 10^6 mutants in the 7500 base-long

genome might be generated, indicating every nucleotide in the genome might have nucleotide alterations. Most of the mutants with amino acid change(s) might not be able to replicate due to dysfunction of viral protein, but some of them would be able to replicate in the presence of favipiravir. We observed 53 transitions and one transversion with 17 amino acid substations in 25,002 bases of the RNA polymerase region of replication-competent viruses (data not shown) and thus every amino acid might have had amino acid changes. We expected the isolation of favipiravir-resistant mutants based on this assumption but isolated only limited number of susceptibility variants and no favipiravir-resistant mutants.

Favipiravir-resistant mutants have not been isolated from influenza virus,[18,26] chikungunya virus,[9] or poliovirus in this study. We therefore concluded that a mutation leading to resistance would be lethal to the RNA-dependent RNA polymerase and not isolation of a favipiravir-resistant virus. The lack of generation of favipiravir-resistant mutants indicates the excellent profile of this antiviral drug in addition to the potent and broad spectrum of anti-RNA virus activity.

Conflicts of interest

All authors declare no conflicts of interest.

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