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Short Communication

Characterization of Susceptibility Variants of Influenza Virus Grown in the Presence of T-705

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Abstract. T-705 (favipiravir) is a potent inhibitor of RNA polymerases of influenza viruses. Susceptibility variants were isolated during passages in the presence of T-705. Nine variants with 0.4 to 2.1 times the 50% inhibitory concentration for plaque formation of the parent A/PR/8/34 (H1N1) strain had amino acid variations in the PB1, PB2, and PA genes of the RNA polymerase complex. However, the variation patterns in the RNA polymerase complex indicated that T-705 does not work as a mutagen, and resistant mutants were not isolated, possibly because a mutation leading to resistance would be lethal to the RNA polymerase function.

Keywords: influenza virus, T-705, favipiravir

T-705 (favipiravir) is a novel anti-influenza agent with a broad spectrum against RNA viruses (1). T-705 inhibits viral RNA-dependent RNA polymerase by inhibiting elongation at the incorporate site as a purine analog (2). This mechanism does not produce mutation in the replicating genome, and accordingly we failed to isolate resistant mutants but did find variants with less susceptibility to T-705.

Some nucleotide analogs are incorporated into the replicating strand of the genome, and these analogs generate mismatched nucleotides as a template in the next complimentary strand formation, resulting in mutation of the further replicating genome. T-705 stops elongation (2, 3) and does not generate a mismatch of the nucleotides in the replicating genome because influenza RNA polymerase has no proofreading activity (4).

Contrasting phenotypes in the generation of mutants by anti-herpetic agents that stop elongation, acyclovir and penciclovir, during the passage in their presence are well known (5). Acyclovir stops elongation at the incorporated site as a guanosine (6), but is removed and re-elongated precisely by the proofreading activity of viral DNA polymerase. Misreadings may accumulate, i.e., a mutation in the guanosine stretch portion as a hot spot in the thymidine kinase gene. In contrast, penciclovir, an active form of famciclovir, stops DNA synthesis at several nucleotides synthesized from the site at which penciclovir is incorporated and does not induce mutation because the terminal is a normal nucleotide and not the target of the proofreading activity.

We used influenza A/PR/8/34 (H1N1) virus for characterization of the susceptible variants and their genome sequences obtained in the presence of T-705 because it is a very frequently used laboratory strain with the best characterized sequence data on the genome containing naturally-occurring variants. Madin-Darby canine kidney (MDCK) cells were grown and maintained in Eagle's minimum essential medium supplemented with 2% and 5% heat-inactivated calf serum, respectively (7). Influenza virus was propagated in MDCK cells in the presence of 2.5 μg/ml of trypsin. MDCK cells in 25 cm^2 flasks were infected with influenza virus, and 28 flasks were independently cultured in the presence of stepwise increasing concentrations of 1, 3, and 5 μg/ml of T-705 for 7 to 11 days with a medium change depending on the spread of the cytopathic effect (CPE). When the CPE developed in a flask containing T-705, an aliquot of 200 μl of the culture supernatant was frozen and thawed three times and inoculated into a new 25 cm^2 flask for further incubation in the presence of T-705. When a culture showed the extensive CPE in the presence of 5 μg/ml of T-705, the infected cells were frozen and thawed three times, and the supernatants were inoculated into MDCK cells in 60-mm plastic dishes with an agarose

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Table 1. IC_{50} of T-705 against various mutants and parental PR8 and amino acid substitutions in various mutants

clone No.	4	7	9	14	15	18	19	21	22
IC_{50} ratio	1.5	0.49	1.19	1.6	2.1	0.40	1.3	1.5	1.5
PB1				M645I	T20I		T20I V709I V715M		
PB2	I463V	V227M	I461V			V212I I260V	T64M A661V	T598I	T598I
PA		R192H				L65F			

overlay containing 1 μg/ml of T-705.

Susceptibility to T-705 was determined by a plaque reduction assay (7). Duplicate cultures of MDCK cells in 60-mm plastic dishes were infected with 100 PFU/0.2 ml of influenza A/PR/8/34 or isolated clones for 1 h at room temperature. Cells were overlaid with 5 ml of nutrient agarose medium containing various concentrations of T-705 and then cultured at 37°C for 2 to 3 day. 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 T-705 for 50% plaque reduction (IC_{50}) were determined from a curve relating the plaque number to the T-705 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 A/PR/8/34 virus assayed simultaneously to minimize the variation of the IC_{50} values between the original and cloned viruses.

Nine susceptibility variant clones were selected from 24 of 28 flasks. These flasks were cultured with increasing concentration from 1 to 5 μg/ml of T-705. The IC_{50} was determined in parallel with the parent virus twice and ranged from 0.59 to 4.11 μg/ml, corresponding to 0.4 to 2.1 times that of the parent virus. Thus, T-705-susceptbility variants were isolated, but T-705-resistant clones with IC_{50} values 3 – 5 times higher were not isolated from virus populations in infected cultures treated continuously with T-705 for a month (Table 1). These variants had plaque sizes similar to those of the parent virus in the absence of T-705. The variations in the change of IC_{50} values were linked to amino acid changes in the PA, PB1, and PB2 genes. The nucleotide sequences of PB1, PB2, and PA were determined in nine clones. RNA extraction was demonstrated by using a QIAamp viral RNA mini kit (QIAGEN GmbH, Germany) and the viral RNA was reverse-transcribed with the Uni12 primer (8) (5′-AGCAAAAGCAGG-3′), using the PrimeScript RT reagent Kit (Takara Bio, Otsu, Shiga). The amplified segments of PB1, PB2, and PA by PCR were sequenced by using the ABI Prism 3130 DNA sequencer.

Nucleotide mutations comprised 33 substitutions in total (3.7 substitutions per clone), and amino acid mutations comprised 16 substitutions in total (1.8 substitutions per clone). Amino acid variations observed in the isolated clones are shown in blue (more sensitive to T-705: lower IC_{50}) or red (less susceptible to T-705: higher IC_{50}) in Fig. 1.

The amino acid variations in the susceptibility variants with slightly higher IC_{50}s were distributed in the functional domains, the PB1 binding and cap binding sites of the PB2 gene and the PA and PB2 binding sites of the PB1 gene, as illustrated in Fig. 1. Thus, the less susceptible variants had the amino acid change in broader regions of the PB1 and PB2 genes. Amino acid changes in the PB1 gene of T20I might be related to the ribavirin resistance associated with the D27N mutation. We did not find any mutant with a mutation in the functional domains of the PB1 gene with transcription and replication functions.

Surprisingly, two clones, 7 and 18, were 2.4 to 2.5 times more sensitive to T-705 than the parent strain, and the amino acid change was not observed in the PB1 gene with transcription and replication functions but was located in the endonuclease active domain in PA of the RNA-dependent RNA polymerase complex (9, 10). This indicated that the PA gene was partly responsible for the T-705 recognition. So far, no T-705-resistant mutant has been isolated in vitro, as reported elsewhere (11), and none was isolated in this study. PB1, PB2, and PA encode transcription and replication activity, cap binding activity, and endonuclease activity, respectively, and form an RNA-dependent RNA polymerase complex. Based on the results with susceptibility variants, T-705 inhibits viral RNA synthesis through the interaction of all three components of the RNA-dependent RNA polymerase complex of influenza virus, PB1, PB2, and PA (9, 10, 12 – 14). The complicated recognition of T-705 by three components of the viral RNA polymerase complex of influenza virus might restrict the three-dimensional change for functioning and might not allow generation of

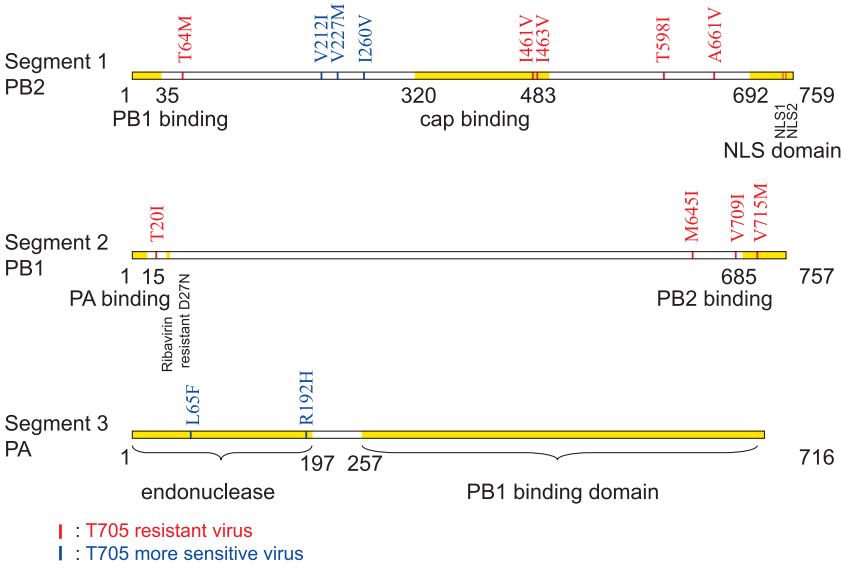

Fig. 1. Locations of amino acid changes in the PB1, PB2, and PA genes of influenza viruses generated during viral replication in the presence of T-705. The functional domains in the gene were modified from published illustrations (9, 10, 12 – 14). Fourteen amino acid variations of 9 susceptibility variants are indicated; variants with lower IC_{50} are shown in blue and those with higher IC_{50} are shown in red. NLS: nuclear localization signal.

T-705-resisitant mutants with a conformational change that is lethal to RNA polymerase function.

Variants with increased susceptibility to antiviral agents have been reported. DNA polymerase mutants of herpes simplex virus and varicella-zoster virus–resistant to acyclovir have acquired lower IC_{50} values to foscarnet or vidarabine (15) and thus the change in amino acids of the viral RNA-dependent RNA polymerase complex might have conferred increased susceptibility of virus clones to T-705.

Baranovich et al. (11) propagated the four strains of H1N1 influenza viruses in the presence and absence of T-705 and observed that T-705 treatment enriched G→T transversion mutations by comparison with non-treated viruses in the culture. Their sequence analysis was performed from viral RNA in the culture supernatant including non-infectious viruses. They speculated that T-705 induced a high rate of mutation that generated a nonviable viral phenotype because the transversion was observed in 34 of 83 substitutions (41%). In contrast, we sequenced the cloned replication-competent virus and observed one transversion (T→G) in 33 substitutions (3%) in our variants, indicating that these substitutions might be due to a naturally occurring transition and not induced by T-705. Moreover, T-705 ribosyl triphosphate terminates chain elongation at the incorporated site and thereby inhibits RNA synthesis without inducing a mismatched substitution (2, 3). Therefore T-705 would not be a mutagen for viral RNA.

Our study indicated that amino acid changes occurred in the viral RNA-dependent RNA polymerase complex and susceptibility variants, but not resistant viruses, were generated. T-705 inhibits the viral RNA-dependent RNA polymerase complex by terminating elongation at the incorporated site (2, 3), resulting in inactivation of the synthesizing strand; it does not cause mismatch of nucleotides because the lack of proofreading activity of viral RNA polymerase complex (4) causes no further RNA synthesis at the incorporated site, indicating that T-705 is not a mutagen. Our result is consistent with the mechanism. The variations observed were not specific for nucleotide species and had no hot spot of variation, indicating that these variations occurred randomly and were not caused by T-705. The results obtained by A/PR/8/34 virus would be true to wild-type viruses including seasonal influenza viruses. In conclusion, T-705 allowed the appearance of susceptibility variants to T-705 but not T-705-resistant mutants, possibly because a mutation leading to resistance would be lethal to the RNA polymerase function.

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