# Investigation of Treatment-Emergent Amino Acid Substitutions in Nonstructural Protein 5 from Ph2a Study of Ensitrelvir, a Novel Oral SARS-CoV-2 3C-Like Protease Inhibitor


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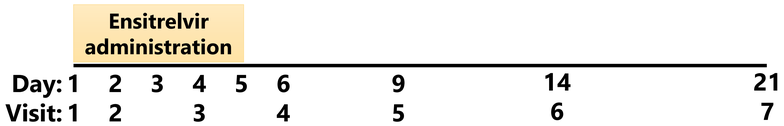
## Background

- Ensitrelvir is a novel oral SARS-CoV-2 3C-like protease (3CL$^{pro}$) inhibitor, and under late clinical development stage for COVID-19 disease.
- In Ph2a and Ph2b studies, ensitrelvir demonstrated rapid decline of viral titer and viral RNA compared with placebo, and tolerability.
- To investigate treatment-emergent amino acid substitutions (TEAASs), we analyzed viral RNA sequences of nonstructural protein 5 (NSP5), the target of ensitrelvir from Ph2a study in Japan.

## Method

- In Ph2a study (28/9/2021~1/1/2022), 47 patients with confirmed SARS-CoV-2 infection (mainly δ strain) were randomized 1:1:1 to ensitrelvir with the loading dose on Day 1/maintenance dose on Day 2-5 (375/125 mg or 750/250 mg), or placebo.
- TEAASs were defined as novel amino acid substitutions identified after treatment with ensitrelvir. NSP5 sequence analysis was performed with direct Sanger sequencing using nasopharyngeal swab samples from 34 patients with ensitrelvir at Day 1, 6, 9, 14, and 21 (V1, 4, 5, 6, and 7) with allowances.
- Infectious viral titer was measured by virus-induced cytopathic effects in VeroE6/TMPRSS2 cells using nasopharyngeal swab samples.
- Viral RNA was quantified by RT-PCR using nasopharyngeal swab samples.
- Drug susceptibility test of ensitrelvir was conducted using SARS-CoV-2 with L87F, T198I, A234S, or H246Y in NSP5 prepared by reverse genetics.




NSP5 sequence analysis: V1, 4, 5, 6, and 7 with allowances.
Viral titration and RT-PCR: V1, 2, 3, 4, 5, 6, and 7 (+day 3 and 5).

## Result

### 1. Mono-TEAAS by NSP5 sequence analysis

| Subject ID | ensitrelvir dose | Visit with TEAAS | TEAAS |
|---|---|---|---|
| 1AA001 | 750/250 mg | V5 | A234S |
| 1EE001 | 375/125 mg | V5 | L87F |
| 1FK007 | 375/125 mg | V5 | H246Y |
| 1HA010 | 750/250 mg | V6 | T198I |

A, alanine; S, serine; L, leucine; F, phenylalanine; H, histidine; Y, tyrosine; T, threonine; I, isoleucine

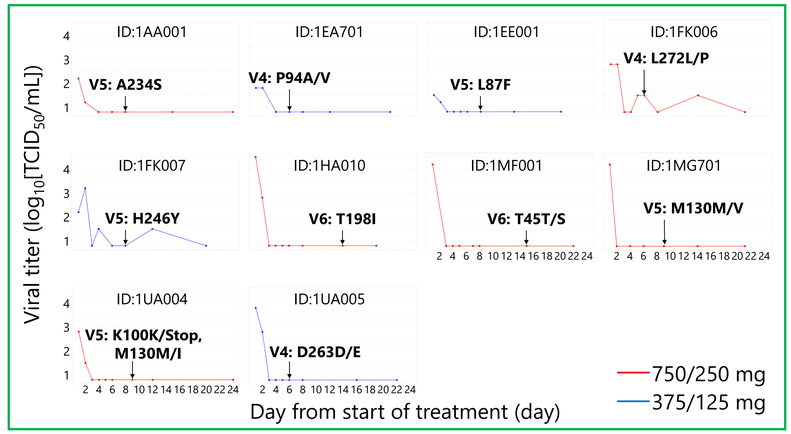
### 2. Mixture of TEAASs by NSP5 sequence analysis

| Subject ID | ensitrelvir dose | Visit with TEAASs | TEAASs |
|---|---|---|---|
| 1EA701 | 375/125 mg | V4 | A94A/V |
| 1FK006 | 750/250 mg | V4 | L272L/P |
| 1MF001 | 750/250 mg | V6 | T45T/S |
| 1MG701 | 750/250 mg | V5 | M130M/V |
| 1UA004 | 750/250 mg | V5 | K100K/Stop, M130M/I |
| 1UA005 | 375/125 mg | V4 | D263D/E |

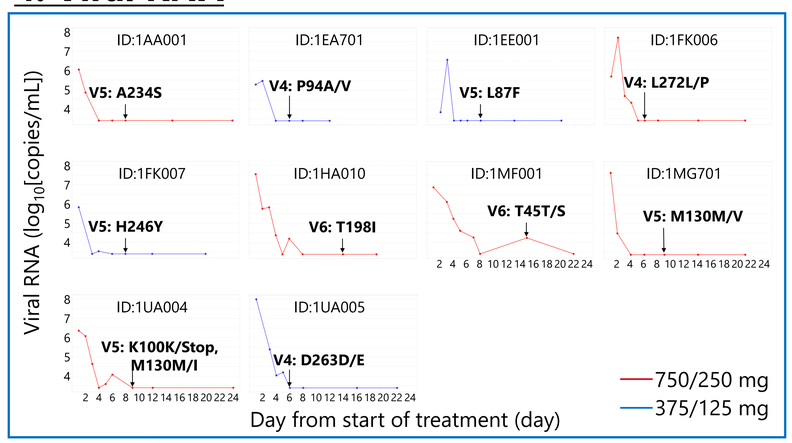
A, alanine; V, valine; L, leucine; P, proline; T, threonine; S, serine; M, methionine; K, lysine; I, isoleucine; D, aspartic acid; E, glutamic acid; Stop, stop codon

**By NSP5 sequence analysis, 10 TEAASs were detected.**

### 3. Viral titer

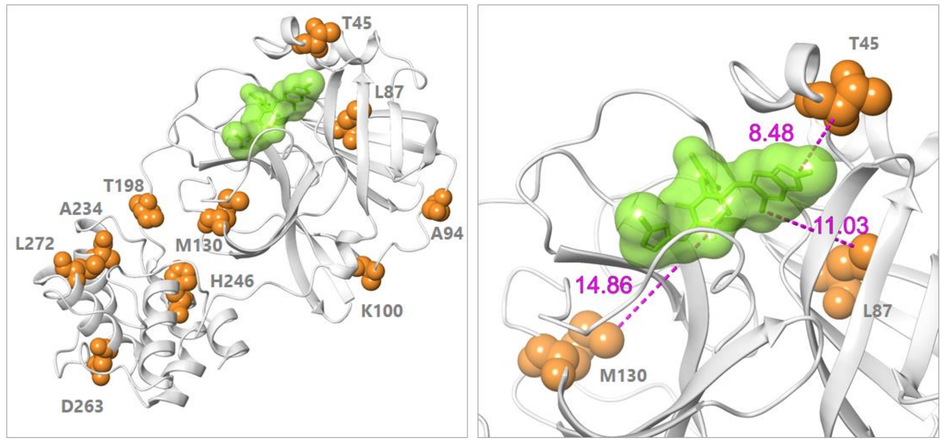



### 4. Viral RNA




**There were no viral rebound.**

### 5. Structural analysis of ensitrelvir and 3CL$^{pro}$




(Left figure) Green, white ribbon, and orange indicate ensitrelvir, 3CL$^{pro}$, and TEAASs, respectively. The words in the figure indicate TEAASs.
(Right figure) The right figure is enlarged around the ensitrelvir binding site in the left figure. The digits in the figure indicate the distance (Å) from ensitrelvir to amino acids. The amino acids with a heavy atom distance of less than 5Å from ensitrelvir were defined as the binding amino acids.

**TEAASs detected in the study are located outside of the active center of 3CL$^{pro}$ which is the binding site of ensitrelvir.**

### 6. Drug susceptibility test using RG virus

| strain | $EC_{50}$ ± SD (μM) | | Fold change | |
|---|---|---|---|---|
| | ensitrelvir | remdesivir | ensitrelvir | remdesivir |
| rgSARS-CoV-2 (wild-type) | 0.21 ± 0.04 | 2.2 ± 0.1 | - | - |
| rgSARS-CoV-2 (nsp5/L87F) | 0.19 ± 0.03 | 1.0 ± 0.0 | 0.92 | 0.47 |
| rgSARS-CoV-2 (nsp5/T198I) | 0.31 ± 0.08 | 1.5 ± 0.0 | 1.5 | 0.72 |
| rgSARS-CoV-2 (nsp5/A234S) | 0.36 ± 0.09 | 2.5 ± 0.1 | 1.7 | 1.2 |
| rgSARS-CoV-2 (nsp5/H246Y) | 0.19 ± 0.03 | 2.0 ± 0.0 | 0.90 | 0.93 |

Fold change = $EC_{50}$ against each mutant strain / $EC_{50}$ against wild-type strain
Data are expressed as the mean ± SD in 3 independent experiments.

**L87F, T198I, A234S, and H246Y do not affect the antiviral activity of ensitrelvir (Fold change<2).**

## Conclusion

In Ph2a study, 10 TEAASs were detected. However, results of viral titer and viral RNA, structural information, and drug susceptibility test suggest that **these TEAASs do not have the impact on antiviral efficacy of ensitrelvir.**


## Acknowledge

We acknowledge Prof. Yoshiharu Matsuura (Osaka University, Osaka, Japan) for providing the materials of reverse genetics.