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Travatrelvir, an Inhibitor of SARS-CoV-2 Main Protease now in Phase 1 Clinical Trials: in vitro Drug Resistance Compared to Nirmatrelvir

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Abstract

Inhibitors of the SARS-CoV-2 main protease (Mpro/3CL) provide valuable antiviral therapy options for persons with COVID19. Nirmatrelvir is the only approved Mpro inhibitor in the U.S. and Europe, but it requires coadministration of the CYP450 inhibitor ritonavir. Our investigational Mpro inhibitor travatrelvir, developed with assistance from the Expert Systems accelerator, is more active than nirmatrelvir and, in 5-day preclinical studies, did not require ritonavir co-administration. Here, we report the patterns of resistance to travatrelvir that were observed during in vitro selection for drug-resistant variants, using travatrelvir at 400 nM (100xEC50). There was extensive overlap between the drug resistance variants reported for nirmatrelvir (Iketani, et al, Nature 613:558, 2023) and travatrelvir. However, two major paths to nirmatrelvir resistance, involving P252L and T304I, were not observed after travatrelvir selection. Travatrelvir IC_{50} 4 nM) was better able to inhibit the activity of wild-type Mpro compared with nirmatrelvir (IC_{50} 27 nM) and was better able to inhibit Mpro with the S144E, E166V, T21I/E166V or T21I/A173V mutations compared to nirmatrelvir. The T21I mutation, which represents less than half of the nirmatrelvir resistance mutations, was present in 68% of travatrelvir-resistant variants, but the P252L and T304I mutations, comprising a large portion of nirmatrelvir resistance, were not detected in our study. The patterns of resistance to travatrelvir overlap with reported resistance mutations for nirmatrelvir but important differences were observed. Travatrelvir has an overall advantage for viral inhibition.

Method

In vitro selection for SARS-CoV-2 resistance to travatrelvir in Vero E6 cells

To select for the development of drug resistance against travatrelvir, SARS-CoV-2 (WA1) was cultured in the presence of increasing concentrations of travatrelvir and passaged 10 times. Vero-E6 cells were cultured in 96-well plates until reaching 90% confluency within 24 hours. Following this, travatrelvir (25 nM) was diluted in DMEM medium supplemented with 2% FBS and 1% antibiotics and added to the plates, with six wells left untreated as controls. The plates were then transferred to the BSL3 suite, and each well was inoculated with 100 TCID50 of virus. After 72 hours, cytopathogenic effect (CPE) was observed. This process is repeated for subsequent passages, with supernatant from the previous passage being added to the next, increasing the drug concentration incrementally up to 400 nM. CPE results are recorded on a scale of 1-4, and supernatant from wells of the final passage showing moderate to severe CPE is collected for further analysis, including virus amplification, testing, and RNA sequencing.

Method

Protein expression and purification

The recombinant 3CLpro sequences were synthesized and introduced into pET28b(+) bacterial expression vectors by GenScript Inc (Piscataway, NJ). The expression of the Hisx6-tagged human 3CLpro protein was performed in E. coli BL21 DE3 (Invitrogen). The inoculated culture (50ml) was grown in LB Broth at 37 °C until the A600 reached 0.6 in the presence of 50 mg/L kanamycin. The temperature was then lowered to 25 °C and the expression was induced overnight with 0.5 mM IPTG. The cells were harvested by centrifugation and resuspended in binding buffer containing 5mM DTT, 5mM EDTA, and 0.1% protease inhibitor cocktail (Sigma P8849). Cell lysis was carried out using sonication and cleared at 16000xg for 30 min at 4 °C. The supernatant was processed using the HisCube Ni-INDIGO Mini kit (Cube Biotech) according to protocol with 3 washes with wash buffer (1mM DTT) and 2 elutions with 400ul elution buffer with 1mM DTT. Elutions were pooled and dialyzed overnight at 4 °C in 20mM Tris pH8, 50mM NaCl, 1mM DTT, and 1mM EDTA in a 10K cutoff cassette (Thermo A52971). The dialyzed protein was then concentrated using 10k cutoff centrifugal filter (Millipore UFC801024). Product was aliquoted and stored at -80 °C.

Mpro activity and inhibition assays in vitro

The final concentration of 3CLpro was 5ug/well for WT, S144E, E166V, T21I/E166V, and T21I/A173V. Final concentration of 3CLpro was 2.5ug/well for T21I, L50F, and T21I/L50F. Each inhibitor was resuspended at a series of concentrations in the reaction buffer (20 mM TRIS pH8, 150 mM NaCl, 1 mM DTT, 5% DMSO). 91 µL of inhibitor in reaction buffer was incubated with 5 µL 3CLpro for 20 min at room temperature. Next, 4 µL of the fluorogenic substrate (1mM) in DMSO was added to each well to initiate the reaction at a final concentration of 9% DMSO and 40uM substrate, in duplicate. The fluorescence was monitored at RT with an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a Tecan Spark Cyto plate reader. The mean slope of each fluorescence curve from 0 to 30 min was calculated as the velocity of the corresponding reaction. The percent proteolytic activity in the presence of each drug was calculated as a ratio of mean slope in presence of inhibitor to mean slope in absence of inhibitor. Two independent experiments were performed. In this format, using laboratory-produced Mpro, the IC_{50} values are generally higher than values obtained with standard commercial assay kits.

Results

Identify resistant mutations to travatrelvir in Vero E6 cells

The frequency of different mutations was calculated and illustrated in Figure 1. The T21I mutation, found in fewer than half of nirmatrelvir-resistant variants, occurred in 68% of travatrelvir-resistant strains, while the P252L and T304I mutations, common in nirmatrelvir resistance, were absent in our study.

Results

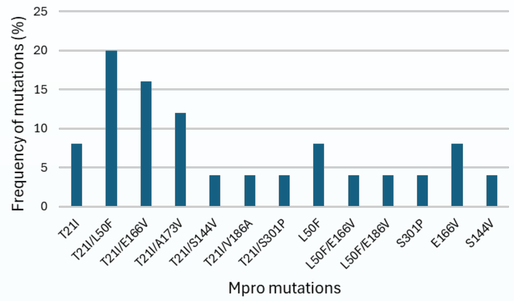

Figure 1: Distribution of travatrelvir resistance in Vero E6 cells. Values (%) indicate the proportion of individual genotypes among 25 independent virus cultures (400 nM travatrelvir).

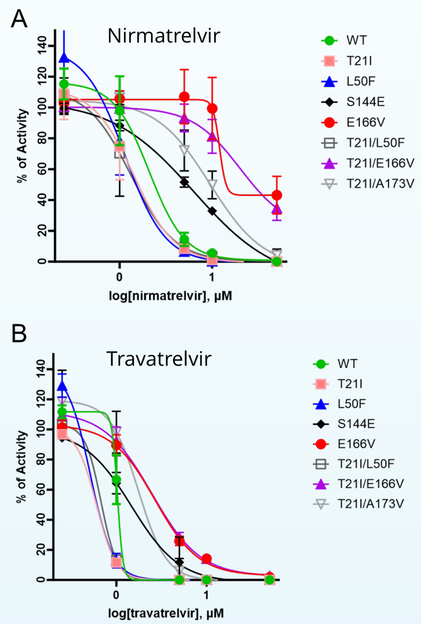

Figure 2: Dose-dependent inhibition of wild-type or mutant Mpro activity by nirmatrelvir or travatrelvir.

Table 1: IC_{50} (µM) Comparisons of Nirmatrelvir and Travatrelvir Against Wild-type and Drug-resistant Mpro Proteins

	WT	T21I	L50F	S144E	E166V	T21I/L50F	T21I/E166V	T21I/A173V
Nirmatrelvir	2.084	1.423	1.14	6.328	12.01	1.319	20.13	9.504
Travatrelvir	1.028	0.601	0.5138	1.474	2.691	0.6771	2.477	1.754

Results

To evaluate the resistance profiles of various mutant Mpro proteins to travatrelvir and nirmatrelvir, we expressed and purified key mutant Mpro variants, including T21I, L50F, S144E, E166V, T21I/L50F, T21I/E166V, T21I/A173V, alongside a wild type control. Utilizing a FRET assay method, we determined the IC50 values of nirmatrelvir (Figure 2A) and travatrelvir (Figure 2B) against these mutant Mpro proteins. We quantified the fold change in inhibition of Mpro activity for both drugs, by comparing wild type and mutant Mpro. Notably, travatrelvir exhibited greater activity compared to nirmatrelvir against wild type Mpro and showed increased activity against Mpro variants with the S144E, E166V, T21I/E166V, or T21I/A173V mutations (Table 1).

Conclusions

This study highlights the potential of travatrelvir, a novel ritonavir-independent Mpro inhibitor, and addresses a critical limitation of existing compounds including nirmatrelvir. Using in vitro selection for drug-resistant variants, we noted overlap between travatrelvir-resistance mutations and published data for nirmatrelvir-resistance mutants. However, there were notable differences between the data sets, including the absence of P252L and T304I mutations after travatrelvir selection, although these mutations comprise a substantial portion of nirmatrelvir resistance in vitro. Travatrelvir exhibited greater activity compared to nirmatrelvir against wild type Mpro and remained active against Mpro variants with specific mutations (S144E, E166V, T21I/E166V, or T21I/A173V) that are associated with nirmatrelvir resistance. While the T21I mutation, prevalent among nirmatrelvir-resistant variants, was present in 68% of travatrelvir-resistant variants, the absence of P252L and T304I mutations suggests a potential advantage for travatrelvir in scenarios where these variants are present in the circulating virus pool. Our findings underscore the importance of understanding resistance mechanisms to guide the choice among individual antiviral therapeutics. Travatrelvir's heightened activity and unique resistance profile position it favorably, especially in the presence of specific nirmatrelvir-resistant variants and support its continued development for COVID19 therapy.

Reference

Multiple pathways for SARS-CoV-2 resistance to nirmatrelvir. Sho Iketani, Hiroshi Mohri, Bruce Culbertson, et al., Nature. 2023; 613(7944): 558–564.

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