Molecular basis for antiviral action of EDP-235: A potent and selective SARS-CoV-2 3CLpro inhibitor for the treatment of COVID-19.

Anand Balakrishnan, Archie Reyes, Ruichao Shen, Nalini Bisht, Joyce Sweeney, Rachel Levene, Nicole McAllister, Tessa Cressey, Nathan Manalo, Michael H. J. Rhodin, Michael Vaine, Guoqiang Wang, Yat Sun Or, Bryan Goodwin.

Enanta Pharmaceuticals, Inc. 500 Arsenal Street, Watertown, MA 02472, USA.

Enanta Pharmaceuticals

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BACKGROUND AND AIM

There is an urgent need for oral antiviral therapies that can be administered early during COVID-19 to suppress progression of the disease or for prophylaxis. SARS-CoV-2, the etiologic agent of COVID-19, is a single-strand RNA virus that depends on the enzyme activity of 3C-like protease (3CLpro) for replication within infected cells. 3CLpro is encoded within ORF1ab and is essential for processing the viral polyproteins and production of viral non-structural components. EDP-235 is a novel, highly selective, and potent (IC_{50} 5.7 nM) 3CLpro inhibitor. Here, we evaluate the *in vitro* pharmacology of EDP-235 and elucidate its molecular mechanism of action using enzymology and structural biology.

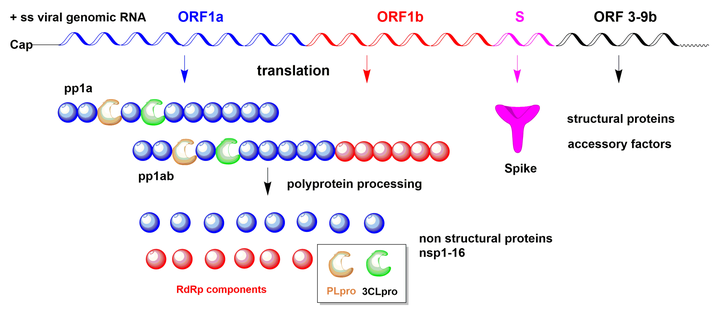


Figure 1. Role of 3CLpro in SARS-CoV-2 viral replication

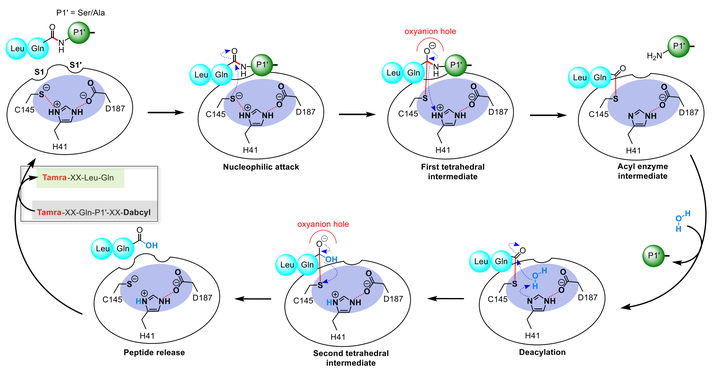


Figure 2. Catalytic mechanism of SARS-CoV-2 3CLprotease

METHODS

Materials. SARS-CoV-2 3CLpro variants, and other coronaviral 3CLpros were expressed and purified from *E coli*. MERS-CoV and SARS-CoV 3CLpro were purchased from R&D Systems. FRET peptides were custom synthesized at Bachem AG, and EDP-235 was synthesized at Enanta. Selectivity assays were performed at Reaction Biology, Inc (Malvern, PA).

Biochemical Assays. 3CLpro activity kinetic assays were assembled in 384w plates and monitored continuously at RT for 0.25-2 h in an Envision reader with fluoresence detection. Assays (20 µL) contained 50 mM HEPES (7.5), 0.01% BSA, 1 mM DTT, 0.01% Triton X-100, 1-100 nM 3CLpro, 1-50 µM FRET peptide, and 0-10 µM EDP-235.

RESULTS

EDP-235 is a potent SARS-CoV-2 3CLpro inhibitor

SARS-CoV-2 Lineage	WHO Classification	ORF1a Mutation	3CLpro Mutation	EDP-235 IC_{50} (nM)
A	n/a	-	-	5.8 ± 3.7
B.1.351	Beta	K3353R	K90R	2.8 ± 0.9
B.1.351.2	Beta	K3353R/A3456V	K90R/A193V	5.4 ± 1.0
P.2	Zeta	L3468V	L205V	3.4 ± 1.0
B.1.617.3	n/a	A3457S	A194S	5.7 ± 0.5
B.1.1.318	n/a	T3284I	T21I	2.0 ± 0.1
C.36.3, C.37	n/a	G3278S	G15S	4.7 ± 2.5
B.1.1.529	Omicron	P3395H	P132H	4.1 ± 0.8

Table 1. EDP-235 is a potent inhibitor of SARS-CoV-2 3CLpro variants. The 3CLpro sequence from the SARS-CoV-2 B.1.617.2 (Delta) lineage is identical to the ancestral A lineage. IC_{50} = half-maximal inhibitory concentration; Data are mean from at least three individual determinations.

RESULTS

EDP-235 is a potent pan-coronaviral 3CLpro inhibitor

Virus	3CLpro Assay IC_{50} (nM)	Live Virus Assay				
		Cell Type	Endpoint	EC_{50} (nM)	CC_{50} (nM)	SI
SARS-CoV-2	**5.8**	Vero E6*	CPE	**5.1**	>10,000	>1,960
HCoV-229E	**5.4**	MRC-5	CPE	**3.6**	>50,000	>13,889
HCoV-HKU1	**3.8**	-	-	-	-	-
HCoV-NL63	**1.8**	LLC-MK2	RT-qPCR	**6.1**	-	-
HCoV-OC43	**3.4**	HCT-8*	RT-qPCR	**56**	-	-
SARS-CoV	**1.9**	Vero E6*	CPE	**24**	>3,000	>125
MERS-CoV	**70**	Vero 76	CPE	**150**	>26,000	>173
			Viral Yield	**130 [EC_{90}]**	>26,000	>200

Table 2. 3CLpro inhibition and antiviral activity of EDP-235 against coronaviruses. *Assay performed in the presence of a P-glycoprotein inhibitor (CP-100356, 2 µM) to prevent transporter-mediated efflux. IC_{50} = half-maximal inhibitory concentration. HCoV-229E, HCoV-HKU1, HCoV-OC43, HCoV-NL63 = human coronavirus 229E, HKU1, OC43, and NL63, respectively; SARS-CoV = severe acute respiratory syndrome; MERS-CoV = Middle East respiratory syndrome. Vero 76 and Vero E6 cells are derived from African green monkey kidney epithelia, MRC-5 are human lung fibroblasts, HCT-8 are derived from a human ileocecal adenocarcinoma, and LLC-MK2 are Rhesus monkey epithelial cells.

EDP-235 is a selective inhibitor of SARS-CoV-2 3CLpro

Host proteases in viral entry

IC_{50} > 100 µM; SI > 17,000

- ACE 2
- Cathepsin B
- Cathepsin L
- TMPRSS 2
- Furin
- Trypsin

Target	EDP-235 IC_{50} (µM)
Caspase 2	4.6
Caspase 3	4.2
Caspase 6	2
Caspase 7	4.7
Caspase 8	22.5
Caspase 9	4.7
Caspase 14	9
Cathepsin K	18.5
SARS-CoV-2 3CLpro	0.0058
Selectivity Index	**> 340**

Table 3. EDP-235 provides a high degree of selective inhibition of SARS-CoV-2 3CLpro over human proteases. Selectivity Index (SI) = $IC_{50}^{off\text{-}target}$/ IC_{50}^{3CLpro}

Kinetics of SARS-CoV-2 3CLpro inhibition by EDP-235

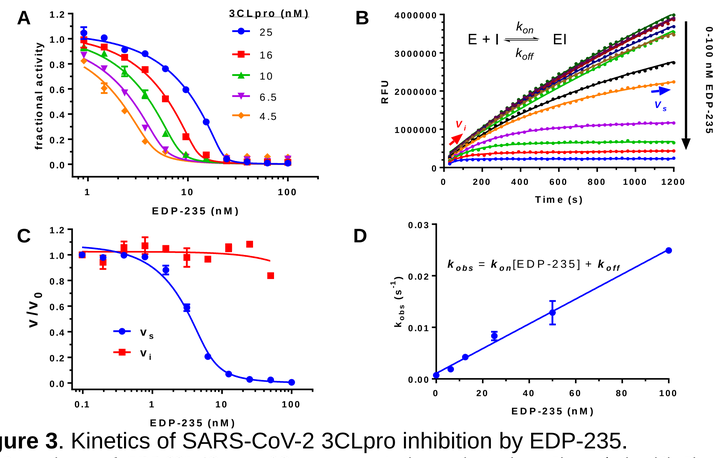
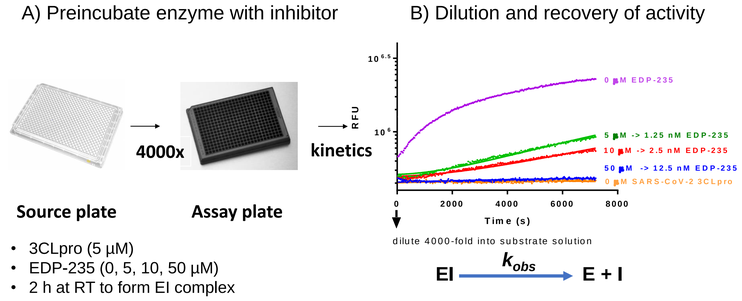


Figure 3. Kinetics of SARS-CoV-2 3CLpro inhibition by EDP-235. (A) Dependence of EDP-235 IC_{50} on 3CLpro concentration and Morrison Fit analysis. (B) Time dependence of 3CLpro inhibition by EDP-235. (C) Second order plots of (C) velocities and (D) k_{obs} versus EDP-235. Representative data are mean ± SD of triplicates (N=3)

EDP-235 is a reversible inhibitor of SARS-CoV-2 3CLpro

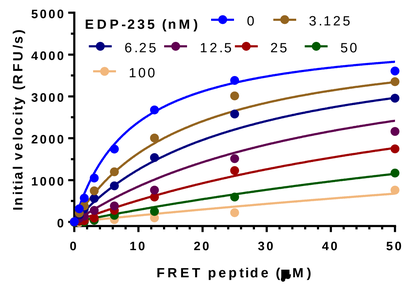


- 3CLpro (5 µM)
- EDP-235 (0, 5, 10, 50 µM)
- 2 h at RT to form EI complex

Figure 4. Jump dilution reversibility test for 3CLpro inhibition by EDP-235. (A) Jump dilution experimental setup and conditions. (B) Progress curves of 3CLpro activity after 4000-fold dilution into substrate solution. Representative data are mean of triplicate curves (N=3)

RESULTS

EDP-235 is a substrate competitive inhibitor of 3CLpro



Parameter	Value
IC_{50}	5.8 nM
K_i	3.0 ± 1.6 nM
k_{on}	(2.6 ± 1.6) x 10^5 $M^{-1}s^{-1}$
k_{off}	(8.7 ± 2.6) x10^{-4} s^{-1}
Residence time	~ 19 min
Mechanism	Slow onset, slow-off rate, reversible, Substrate competitive

Figure 5. EDP-235 is a substrate competitive inhibitor of 3CLpro. (Left) EDP-235 is a substrate competitive inhibitor of SARS-CoV-2 3CLpro. Representative data are mean ± SD of triplicates (N=3) (Right) Summary of kinetic parameters for 3CLpro inhibition by EDP-235. Data are mean ± SD of 3 experiments.

EDP-235 binds to the active site of SARS-CoV-2 3CLpro

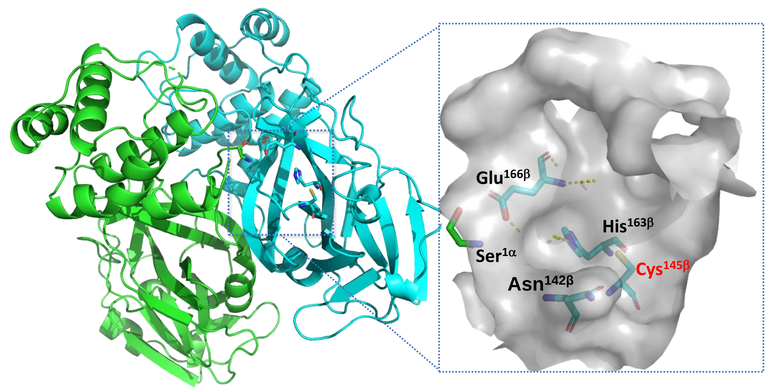


Figure 6. EDP-235 binds to the active site of SARS-CoV-2 3CLpro. (Left) SARS-CoV-2 3CLpro crystallized as a dimer in presence of an EDP-235 analog. (Right) EDP-235 analog bound at the active site of SARS-CoV-2 3CLpro and makes strong interactions with conserved active site residues.

Preclinical Profile of EDP-235: Potentially Best in Class

Preclinical Properties		EDP-235[1]	PF-07321332[2]
Mechanism		**Protease**	Protease
Potency	Enzyme IC_{50} (nM)	**5.8**	19
	Vero Cell EC_{50} (nM)	**5.1**	75
Barrier to Resistance		HIGH	not determined
Oral Bioavailability[3]		**95%**	34 – 50%
Lung Penetration[4]		**4.1**	0.8[5]
Projected Efficacious Dose		**100 – 500mg QD**	250mg/100mg **RTV** q12h

Table 4. Summary of preclinical properties of EDP-235. (1) Jiang *et al.*, ISIRV Poster #120, Oct 19, 2021 (2) Owen *et al.*, medRxiv, July 2021; Pfizer 2Q2021 earnings presentation. (3) Oral bioavailability in rats for EDP-235, PF-07321322 (4) AUC lung to plasma ratio in rats for EDP-235, PF-07321332. (5) Data for PF-07321332 generated by Enanta

CONCLUSIONS

- EDP-235 is a potent inhibitor of SARS-CoV-2 3CLpro and shows potent antiviral activity against SARS-CoV-2 variants of concern and other pathogenic human coronaviruses.
- EDP-235 showed a high degree of selectivity (SI >300-fold) towards SARS-CoV-2 3CLpro compared to human proteases.
- EDP-235 is a time-dependent reversible inhibitor of SARS-CoV-2 3CLpro with an on-target residence time of ~19 min.
- EDP-235 is a substrate competitive inhibitor which binds at the active site of SARS-CoV-2 3CLpro and interacts with conserved active site residues. These interactions may provide a high barrier to resistance development.
- EDP-235's preclinical profile suggests the potential for a best in class, oral antiviral treatment for COVID-19.

ACKNOWLEDGEMENTS

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