Pharmacological Research 223 (2026) 108063

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Evaluation of *in vitro* antiviral activity against different SARS-CoV-2 variants of a protease PROTAC degrader

ARTICLE INFO

Keywords:
SARS-CoV2
Protease inhibitors
PROTAC
In vitro

Dear Editor,

The SARS-CoV-2 main protease (Mpro). plays a central role in viral replication, making infected-cell systems a relevant platform for evaluating antiviral candidates under biologically meaningful conditions. Currently approved inhibitors, such as nirmatrelvir/ritonavir are effective but limited by drug–drug interactions [1] and frequent dosing requirements [2]. These constraints motivate the development of alternative antiviral strategies that can be assessed directly in vitro using SARS-CoV-2–infected cellular models.

In this context, we employed SARS-CoV-2–infected VERO E6 cells as an experimental system to characterize antiviral responses, cytopathic effects. As a proof-of-concept compound, we selected FT235, a peptidomimetic PROTAC previously developed at University of Florence to induce Mpro degradation [3]. FT235 combines a GC-376–derived dipeptidyl warhead targeting MPro in the active site with a pomalidomide moiety, enabling the recruitment of cereblon and proteasomal clearance of the viral protease.

Following the previous investigation of the in-vitro functionality of FT235 [3], this compound was used as a suitable model for evaluating and optimizing our infected-cell experimental system. The data available in the literature, together with the growing interest in PROTAC-based antiviral strategies [4], make FT235 a useful example for exploring the potential of PROTAC technology in the context of SARS-CoV-2 infection.

Accordingly, we investigated the *in vitro* cytotoxicity profile of FT235. For this purpose, VERO E6 cells, obtained from the American Type Culture Collection, ATCC CRL-1586, Manassas, VA, USA] were employed. Cells were seeded at a density of 1 × 10^5 cells/well and pre-incubated for 24 h before treatment. Serial two-fold dilutions of FT235 were prepared, starting from a maximum concentration of 200 μM. Cell viability was assessed using the luminescent CellTiter-Glo 2.0 assay (Promega, Madison, WI, USA) following the manufacturer's instructions, and luminescence was recorded using a GloMax® Discover multimode microplate reader [REF GM3000 (Promega Corporation, USA)].

The 50 % cytotoxic concentration (CC_{50}) was determined by nonlinear dose–response curve fitting using GraphPad Prism (v.9.0.2). Untreated cells and DMSO-treated cells were used as 0 % and 100 % viability controls, respectively, to normalize the data. Each compound concentration was tested in at least three replicates across a minimum of three independent experiments. Notably, cytotoxicity assays were performed in the presence and absence of the P-glycoprotein (P-gp) inhibitor CP-100356 monohydrochloride (CAS n° 142715-48-8) at 0.5 μM, a concentration corresponding to its reported IC_{50} for MDR1 inhibition [5]. The results showed no detectable cytotoxicity at concentrations up to 100 μM, supporting a low cytotoxic profile for FT235 under the tested conditions (Fig. 1).

The antiviral activity of FT235 was assessed in VERO E6 cells infected with SARS-CoV-2 variants B.1 [EPI_ISL_2472896], BA.5.1 [EPI_ISL_18450047], and JN.1 [EPI_ISL_18863880].

Swabs positivity was evaluated by qPCR, using Luna® Universal One-Step RT-qPCR (New England BioLabs, Ipswich, MA) and SARS-CoV-2 (2019-nCoV) CDC qPCR Probe Assay (Integrated DNA Technologies, Coralville, IA) kits. Whole genome sequences were obtained using a modified version of the ARTIC protocol (https://artic.network/ncov-2019; accessed September 29, 2025) using the Illumina DNA Prep and the IDT ILMN DNA/RNA Index kit (Illumina, San Diego, CA, USA). Sequencing was performed on Illumina Miseq platform (Illumina Reads were mapped and aligned to the reference genome obtained from GISAID (https://www.gisaid.org/) [NC_045512.2] using Geneious Prime software v. 2025.2 (Biomatters, Auckland, New Zealand) (http://www.geneious.com). Obtained sequences were classified using the Pangolin COVID-19 Lineage Assigner v. 4.3 (https://pangolin.cog-uk.io/) and Nextclade v. 2.14.1 (https://clades.nextstrain.org/).

Cells were pre-incubated for 1 h with two-fold serial dilutions of FT235, followed by infection at MOI 0.01 and 72 h incubation. Assays were performed with or without the P-glycoprotein (P-gp) inhibitor CP100356 (0.5–1.5 μM) to assess the contribution of efflux mechanisms. FT235 showed moderate activity against the ancestral B.1 strain (IC_{50} = 187.99 ± 20.78 μM), which improved to 77.52 ± 3.21 μM in the presence of CP100356. Significant differences were observed at 100, 50, and 25 μM ($p < 0.0001$–0.0068), whereas no differences were detected at lower concentrations.

FT235 efficacy was partially reduced at 1.0–1.5 μM CP100356

https://doi.org/10.1016/j.phrs.2025.108063
Received 1 November 2025; Received in revised form 27 November 2025; Accepted 8 December 2025
Available online 9 December 2025

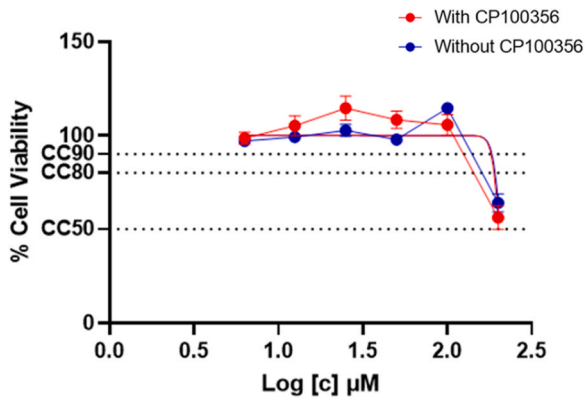

Fig. 1. FT235 cytotoxicity with and without P-gp inhibitor.

compared to 0.5 µM, although most differences remained statistically significant ($p < 0.0001$–0.0156, IC_{50} = 96.13–120.06 µM), although differences remained significant in most cases ($p < 0.0001$–0.0156) (Fig. 2A). Based on these results, 0.5 µM CP100356 was selected for subsequent experiments, as this concentration showed the highest cell viability at the first FT235 concentration (100 µM) compared to 1.0 and 1.5 µM CP100356 (statistically significant for both), while no significant differences were observed at lower FT235 concentrations. This allowed the selection of a P-gp inhibitor concentration that optimized cell viability across the full range of FT235 concentrations (Fig. 2B).

Against JN.1, FT235 showed greater potency ($IC_{50} = 44.27 \pm 4.99$ µM without and 18.08 ± 4.08 µM with CP100356), consistent with an enhanced effect upon P-gp inhibition. In contrast, no measurable activity was detected against the Omicron BA.5.1 variant, suggesting that structural changes in Mpro may impair PROTAC efficacy (Fig. 2C). Focusing on the nsp5 region encoding Mpro, catalytic residues were highly conserved across B.1, BA.5.1, and JN.1 variants, with no significant amino acid changes. Nevertheless, mutations in distal or allosteric regions could subtly affect protease conformation and active site accessibility. These structural variations likely contribute to the observed differences in FT235 antiviral activity, with higher potency against JN.1 and reduced efficacy against B.1 and BA.5 SARS-CoV-2 variants indicating that PROTAC-mediated degradation depends not only on catalytic site conservation but also on the overall Mpro structure and warhead engagement.

Our study demonstrates that the cell assay established in infected VERO E6 cells provides a robust and informative platform for evaluating antiviral activity under biologically relevant conditions. This experimental setup proved suitable for detecting variant-specific responses, supporting its applicability for the characterization of novel antiviral candidates.

The use of FT235 allowed us to explore the performance of this platform in a real-virus context. Although its antiviral activity displayed variant-dependent differences and remains limited against some lineages, the responses we observed reinforce the need for further investigation of FT235, including pharmacokinetic properties, *in vivo* efficacy, and potential broad-spectrum activity.

By demonstrating the utility of in vitro approach with a peptidomimetic PROTAC, our findings open the way to future studies aimed at confirming the effectiveness of this drug candidate, especially in vivo and in human models.

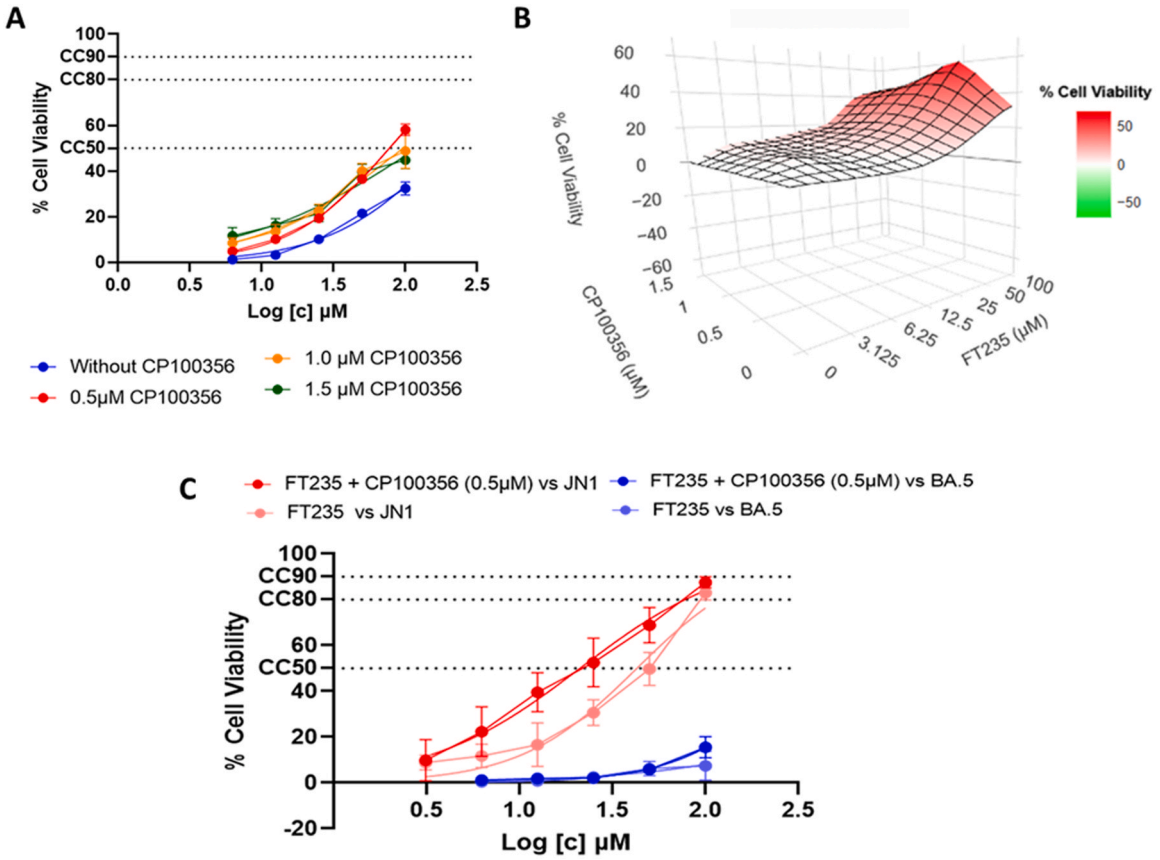

Fig. 2. Antiviral efficacy of FT235. (A) Antiviral activity of FT235 against SARS-CoV-2 B.1 variant. (B) 3D representation of the interaction between FT235 and CP100356 on cell viability, highlighting the optimal dosage of the P-gp inhibitor to maximize the response. (C) Comparison of FT235 antiviral activity against JN.1 and BA.5 with or without P-gp inhibitor.

Materials and methods

VERO E6 cells [also known as Vero C1008, obtained from the American Type Culture Collection, ATCC CRL-1586, Manassas, VA, USA] were used to assess the *in vitro* cytotoxicity profile of FT235. Cells were seeded at a density of $1 \times 10\hat{}5$ cells/well and pre-incubated for 24 h before treatment. Serial two-fold dilutions of FT235 were prepared, starting from a maximum concentration of 200 μM. Cell viability was assessed using the luminescent CellTiter-Glo 2.0 assay (Promega, Madison, WI, USA) following the manufacturer's instructions, and luminescence was recorded using a GloMax® Discover multimode microplate reader [REF GM3000 (Promega Corporation, USA)].

The 50 % cytotoxic concentration (CC_{50}) was determined by nonlinear dose–response curve fitting using GraphPad Prism (v.9.0.2). Untreated cells and DMSO-treated cells were used as 0 % and 100 % viability controls, respectively, to normalize the data. Each compound concentration was tested in at least three technical replicates across a minimum of three independent experiments. Cytotoxicity assays were performed in the presence and absence of the P-glycoprotein (P-gp) inhibitor CP-100356 monohydrochloride (CAS n° 142715-48-8) at 0.5 μM, a concentration corresponding to its reported IC_{50} for MDR1 inhibition.

The antiviral activity of FT235 was assessed in VERO E6 cells infected with SARS-CoV-2 variants B.1 [EPI_ISL_2472896], BA.5 [EPI_ISL_18450047], and JN.1 [EPI_ISL_18863880]. Cells were pre-incubated for 1 h with two-fold serial dilutions of FT235, followed by infection at MOI 0.01 and 72 h incubation. Assays were performed with or without the P-glycoprotein (P-gp) inhibitor CP100356 (0.5–1.5 μM) to assess the contribution of efflux mechanisms.

Swabs positivity was evaluated with the multiplexed RT-qPCR developed by English consortium (https://www.protocols.io/view/multiplexed-rt-qpcr-to-screen-for-sars-cov-2-b-1-1-br9vm966?). Whole genome sequences were obtained using a modified version of the ARTIC protocol (https://artic.network/ncov-2019; accessed September 29, 2025) using the Illumina DNA Prep and the IDT ILMN DNA/RNA Index kit (Illumina, San Diego, CA, USA). Sequencing was performed on Illumina Miseq platform (Illumina Reads were mapped and aligned to the reference genome obtained from GISAID (https://www.gisaid.org/) [NC_045512.2] using Geneious Prime software v. 2025.2 (Biomatters, Auckland, New Zealand) (http://www.geneious.com). Obtained sequences were classified using the Pangolin COVID-19 Lineage Assigner v. 4.3 (https://pangolin.cog-uk.io/) and Nextclade v. 2.14.1 (https://clades.nextstrain.org/).

CRediT authorship contribution statement

Stefano Rusconi: Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Ioana Seravalli:** Writing – review & editing, Visualization. **Simone Ciofi-Baffoni:** Visualization, Methodology, Investigation. **Elena Lenci:** Visualization, Methodology, Investigation. **Andrea Trabocchi:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Annalisa Bergna:** Visualization, Methodology. **Alessia Lai:** Writing – review & editing, Visualization, Methodology, Investigation. **Carla Della Ventura:** Writing – review & editing, Validation. **Arianna Gabrieli:** Visualization. **Mirko Giuseppe Liturri:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

SR received honoraria for presentations and scientific advice from MSD, ViiV Healthcare, Menarini, AstraZeneca, and Gilead Sciences and research grants for his institutions from ViiV Healthcare and Gilead Sciences.

Acknowledgments

Supported by Intesa Sanpaolo (B/2021/0212, B/2022/0209) EU NextGenerationEU–PNRR (THE, ECS00000017, CUP B83C22003920001), MUR Departments of Excellence (DICUS 2.0), UniFI Chemistry "Ugo Schiff" and an unrestricted research grant from Fondazione Sodalitas, Milan, to SR.

Data availability

Data will be made available on request.

References

[1] F. Girardin, O. Manuel, C. Marzolini, T. Buclin, Evaluating the risk of drug-drug interactions with pharmacokinetic boosters: the case of ritonavir-enhanced nirmatrelvir to prevent severe COVID-19, Clin. Microbiol. Infect. 28 (8) (2022) 1044–1046, https://doi.org/10.1016/j.cmi.2022.03.030 (PMID: 35358684; PMCID: PMC8958820).

[2] N.W. Lange, D.M. Salerno, D.L. Jennings, J. Choe, J. Hedvat, D.B. Kovac, J. Scheffert, T. Shertel, L.E. Ratner, R.S.Jr. Brown, M.R. Pereira, Nirmatrelvir/ritonavir use: managing clinically significant drug-drug interactions with transplant immunosuppressants, Am. J. Transpl. 22 (7) (2022) 1925–1926, https://doi.org/10.1111/ajt.16955 (PMID: 35015924).

[3] D. Grifagni, E. Lenci, A. De Santis, A. Orsetti, C.G. Barracchia, F. Tedesco, R. Bellini Puglielli, F. Lucarelli, A. Lauriola, M. Assfalg, F. Cantini, V. Calderone, D. Guardavaccaro, A. Trabocchi, M. D'Onofrio, S. Ciofi-Baffoni, Development of a GC-376 based peptidomimetic PROTAC as a degrader of 3-chymotrypsin-like protease of SARS-CoV-2, ACS Med. Chem. Lett. 15 (2) (2024) 250–257, https://doi.org/10.1021/acsmedchemlett.3c00498 (PMID: 38352832; PMCID: PMC10860180).

[4] C. Wei, Y. Li, L. Guo, Z. Shao, H. Diao, Development of peptidomimetic PROTACs as potential degraders of 3-chymotrypsin-like protease of SARS-CoV-2, Int. J. Mol. Sci. 26 (8) (2025), https://doi.org/10.3390/ijms26083903 (PMID: 40332755; PMCID: PMC12027948).

[5] ⟨https://www.medchemexpress.com/cp-100356-hydrochloride.html?srsltid=AfmBOopbB7qm94dvOO_Z7PItvM8rESWiOhxjKiMl_RQgjTyZMQZdBpJd⟩.

Mirko G. Liturri, A. Bergna, A. Lai, C. Della Ventura, A. Gabrieli, I. Seravalli
Infectious Diseases Research Lab, DIBIC, University of Milan, Italy

S. Ciofi-Baffoni, E. Lenci, A. Trabocchi
Dipartimento di Chimica "Ugo Schiff" (DICUS), University of Florence, Italy

S. Rusconi*
Infectious Diseases Research Lab, DIBIC, University of Milan, Italy
Infectious Diseases Unit, Legnano General Hospital, ASST Ovest Milanese, Legnano, MI, Italy

* Corresponding author at: Infectious Diseases Research Lab, DIBIC, University of Milan, Italy.
E-mail address: stefano.rusconi@unimi.it (S. Rusconi).