In vitro evaluation of the activity of teriflunomide against SARS-CoV-2 and the human coronaviruses 229E and OC43

Paul Lang a,*, Svend S. Geertsen b, Alex L. Lublin b, Michelle C. Potter b, Tatiana Gladysheva a, Jill S. Gregory a, Pascal Rufi c

a Sanofi, 153/211 Second Avenue, Waltham, MA, 02451, USA
b Sanofi, 450 Water Street, Cambridge, MA, 02141, USA
c Sanofi, 1 Av. Pierre Brussels, 91380, Chilly-Mazarin, France

ARTICLE INFO

Keywords:
Antiviral
Coronavirus
COVID-19
Human cell line
SARS-CoV-2
Teriflunomide

ABSTRACT

Previous data have suggested an antiviral effect of teriflunomide, including against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the agent underlying the ongoing COVID-19 pandemic. We undertook an in vitro investigation to evaluate the inhibitory activity of teriflunomide against SARS-CoV-2 in a cell-based assay. Teriflunomide was added to Vero (kidney epithelial) cells that had been infected with SARS-CoV-2. A nucleocapsid immunofluorescence assay was performed to examine viral inhibition with teriflunomide and any potential cytotoxic effect. The 50% effective concentration (EC_{50}) for teriflunomide against SARS-CoV-2 was 15.22 μM. No cytotoxicity was evident for teriflunomide in the Vero cells (i.e., the 50% cytotoxic concentration [CC_{50}] was greater than the highest test concentration of 100 μM). The data were supported by additional experiments using other coronaviruses and human cell lines. In the SARS-CoV-2-infected Vero cells, the produg leflunomide had an EC_{50} of 16.49 μM and a CC_{50} of 54.80 μM. Our finding of teriflunomide-mediated inhibition of SARS-CoV-2 infection at double-digit micromolar potency adds to a growing body of evidence for a broad-ranging antiviral effect of teriflunomide.

1. Introduction

Despite the availability of an increasing number of approved vaccines, the COVID-19 pandemic continues to present a global health emergency. A key component of efforts to reduce COVID-19-associated morbidity and mortality is the development of successful antiviral treatments. An approach to address this challenge is to examine repurposing currently approved therapies with possible antiviral properties, for use against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the RNA virus that causes COVID-19 [1].

Teriflunomide is a disease-modifying therapy used for the treatment of relapsing forms of multiple sclerosis (MS). The hypothesized mechanism of action for teriflunomide entails targeting activated B and T lymphocytes involved in MS pathogenesis through inhibition of the enzyme dihydroorotate dehydrogenase, which is essential for de novo pyrimidine biosynthesis [2]. In addition, preclinical studies have suggested that teriflunomide exerts inhibitory activity against a range of viruses. Among DNA viruses, teriflunomide has been shown to inhibit virion assembly of herpes simplex virus-1 [3], lytic reactivation and replication of Epstein–Barr virus [4], replication and early gene expression in BK polyomavirus [5], and infection and spread of John Cunningham polyomavirus [6]. Teriflunomide also disrupted the abnormal proliferation of T cells that occurs with infection by the retrovirus human T-lymphotropic virus-1 [7], and reduced production of the RNA viruses human respiratory syncytial virus [8] and Junín virus [9]. Most recently, a potential antiviral effect of teriflunomide on SARS-CoV-2 has been suggested based on in vitro data [10] and supported by a computational binding model of molecular docking [11].

Given the emergence of multiple variants of COVID-19 during the pandemic and the evidence suggesting broad activity of teriflunomide, the evaluation of the activity of teriflunomide against SARS-CoV-2 and the human coronaviruses 229E and OC43 was undertaken in this study.
among multiple species of viruses, we undertook an in vitro investigation of teriflunomide and its prodrug leflunomide using human cell lines infected with SARS-CoV-2, as well as other coronaviruses.

2. Materials and methods

Full details of the materials and methods can be found in the Supplementary Appendix. Briefly, the test compounds for antiviral experiments were teriflunomide and the prodrug leflunomide. Experiments were quality controlled with the reference compounds remdesivir (used both in a blinded assay and as an internal reference compound), lopinavir, and chloroquine.

Vero (kidney epithelial) cells infected with SARS-CoV-2 were assessed for nucleocapsid immunofluorescence combined with cytopathic effect, evaluating protection from either host cell lysis or host cell death due to inability to replicate. Compounds diluted in cell assay medium (3-fold [test compounds] or 2-fold [reference compounds] serial dilutions to 10 different concentrations; in duplicate wells) were added to Vero cells that had been seeded in cell culture plates (12,000 cells/well). Cells were then infected with SARS-CoV-2 at a multiplicity of infection of 0.0125.

Additional experiments evaluated cytopathic effect and quantitative real-time polymerase chain reaction (qPCR) for the human coronavirus (HCoV) strains 229E (prototype alpha coronavirus) and OC43 (prototype beta coronavirus), which were propagated in MRC5 (human fetal lung fibroblast) and Hu7 (hepatoma tissue) cells, respectively. Compounds diluted in cell assay medium (3-fold serial dilutions to 8 different concentrations; in duplicate wells) were added to cells that had been seeded in cell culture plates (MRC5, 20,000 cells/well; Hu7, 8000 cells/well). MRC5 cells were infected with 200 TCID$_{50}$ of HCoV 229E and Hu7 cells were infected with 100 TCID$_{50}$ of HCoV OC43 (tissue culture infectious dose [TCID$_{50}$] is the titer of viral stock at which cytopathic effect develops in only half of replicate cultures). Methods for the qPCR followed the protocol previously described by Wang et al. [12].

For all experiments, cell controls (not infected with a virus and not treated with a compound) and virus controls (infected with a virus and not treated with a compound) were tested in parallel. Antiviral activity of compounds is expressed as percentage inhibition: the 50% effective concentration (EC$_{50}$) is the concentration of compound at which 50% of maximal effect is observed. Cytotoxicity is expressed as percentage viability, i.e. the 50% cytotoxic concentration (CC$_{50}$).

3. Results and discussion

The inhibition and cytotoxicity curves for teriflunomide in the SARS-CoV-2–infected Vero cells are presented in Fig. 1. The EC$_{50}$ was 15.22 μM for teriflunomide and 16.49 μM for the prodrug leflunomide. Similar EC$_{50}$ values have been reported in the literature [10]. Furthermore, the concentrations of teriflunomide assessed in our study are of clinical relevance, with a previous study describing a teriflunomide plasma concentration of 130 μM (35 mg/L) in a patient with MS [13]. These findings are also consistent with a recent meta-regression of observational studies of patients with MS (N = 5634; mean age, 41.8 years) and COVID-19 (15.5% hospitalized), which suggested a protective effect of teriflunomide on COVID-19-related lethality ($\beta$ = –0.11, $P$ = 0.035) [14].

Teriflunomide showed no obvious cytotoxicity in the Vero cells (i.e., its CC$_{50}$ value was greater than the highest test concentration of 100 μM). The cytotoxicity was lower than that of leflunomide (CC$_{50}$ 54.80 μM), which was previously reported to improve both the SARS-CoV-2 clearance rate and the hospital discharge rate in a pilot study of 27 hospitalized patients with refractory COVID-19 [15]. Immunofluorescence images of the SARS-CoV-2–infected Vero cells subjected to increasing doses of teriflunomide are presented in Supplementary Fig. 1.

The reliability of the experiments with teriflunomide was confirmed by the effects seen for the reference compounds remdesivir, lopinavir, and chloroquine. In particular, remdesivir showed obvious inhibitory activity against SARS-CoV-2 with an EC$_{50}$ of 0.56 μM, as well as expected effects on cell viability.

The antiviral effect of teriflunomide against SARS-CoV-2 in our study was supported by results in the other cell lines tested, which were derived from either human lung fibroblasts or human hepatoma tissue as an experimental substitute for hepatocytes (Table 1). With the exception of the HCoV OC43 cytopathic effect assay, the alpha or beta coronavirus infection in these cells was inhibited by teriflunomide at the concentrations tested. Teriflunomide also showed no obvious cytotoxicity in

![Fig. 1. Activity and cytotoxicity of compounds in the SARS-CoV-2 immunofluorescence assay in Vero cells. Panels A–C present inhibition of viral infection (green curves with dot markers) and assessment of cytotoxicity (blue curves with triangle markers) for the test compounds teriflunomide (A) and leflunomide (B), as well as remdesivir in a blinded assay (C). The two data point markers at each concentration in Panels A–C represent the values from individual wells at that concentration on each of the duplicate plates. Panels D–F present inhibition of viral infection (green curves with dot markers) and assessment of cytotoxicity (blue curves with diamond markers) for the reference compounds chloroquine (D) and lopinavir (E), as well as remdesivir as an internal reference (F). The data point marker at each concentration in Panels D–F represents the mean of values from triplicate wells at that concentration on a single plate (error bars are standard error of the mean [SEM]). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)](image-url)

P. Lang et al.
these additional cell lines, with CC50 consistently >100 μM in the assays. These additional experiments again showed the expected antiviral activity with the reference compound remdesivir, as well as some evidence for reduced cell viability with leflunomide (HCoV OC43 qPCR assay) (Table 1).

A limitation of the present study is the need for caution in interpreting results due to protein binding differences among cell types. Although Vero cells are widely used to assess drug repurposing, a recent study found that antiviral activity observed against SARS-CoV-2 in Vero cells was reproducible in human lung epithelial Calu-3 cells for remdesivir, but not for chloroquine [16]. Therefore, it will be important to confirm our results for teriflunomide against SARS-CoV-2 in another suited cell line, such as human lung epithelial cells.

In conclusion, teriflunomide showed antiviral activity against SARS-CoV-2, with a mild toxicity profile. Continued efforts to develop effective treatments will be critical toward reducing the burden of COVID-19, particularly in patients who are unvaccinated or undervaccinated, and in those whose immune responses generated by vaccines may be suboptimal against emerging variants of SARS-CoV-2.

Funding

This study was funded by Sanofi. Sanofi was involved in the study design; collection, analysis and interpretation of the data; writing the report; and the decision to submit the article for publication.

Author contributions

The study was designed by PL, ALL, MCP, TG, and JSG. Data were collected by PL. Data were verified by ALL. All authors provided interpretation of data and critical revision of the drafts. The final version of the manuscript was approved by all authors, who had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Data sharing

Data are available upon request. Further details on Sanofi’s data sharing criteria, eligible studies, and process for requesting access can be found at: https://www.vivli.org/.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Paul Lang, PhD reports writing assistance was provided by Elevate Scientific Affairs, Envision Pharma Group. Paul Lang, PhD reports a relationship with Sanofi that includes: employment.

Sven S. Geertsen, PhD reports writing assistance was provided by Elevate Scientific Affairs, Envision Pharma Group. Sven S. Geertsen, PhD reports a relationship with Sanofi that includes: employment.

Alex L. Lublin, PhD reports writing assistance was provided by Elevate Scientific Affairs, Envision Pharma Group. Alex L. Lublin, PhD reports a relationship with Sanofi that includes: employment.

Michelle C. Potter, PhD reports writing assistance was provided by Elevate Scientific Affairs, Envision Pharma Group. Michelle C. Potter, PhD reports a relationship with Sanofi that includes: employment.

Patricia Strasser, PhD reports writing assistance was provided by Elevate Scientific Affairs, Envision Pharma Group. Pascal Rufi, MD reports a relationship with Sanofi that includes: employment.

Tatiana Gladysheva reports writing assistance was provided by Elevate Scientific Affairs, Envision Pharma Group. Tatiana Gladysheva reports a relationship with Sanofi that includes: employment.

Jill S. Gregory reports writing assistance was provided by Elevate Scientific Affairs, Envision Pharma Group. Jill S. Gregory reports a relationship with Sanofi that includes: employment.

Pascal Rufi, MD reports writing assistance was provided by Elevate Scientific Affairs, Envision Pharma Group. Pascal Rufi, MD reports a relationship with Sanofi that includes: employment.

Acknowledgements

The authors thank Maria Melanson and Bruno Padrazzi for supporting this study at Sanofi, as well as Bill Aschenbach (an employee of Sanofi when the work was conducted [current affiliation: Keros Therapeutics]) for his contributions to study design and review of the manuscript. Medical writing assistance was provided by Richard J. Hogan, PhD, of Elevate Scientific Solutions, a division of Envision Pharma Group, and editorial and graphics assistance was provided by Elevate Scientific Solutions. This support was funded by Sanofi.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2022.101395.

Table 1

|                  | HCoV 299E CPE assay | HCoV 299E qPCR assay | HCoV OC43 CPE assay | HCoV OC43 qPCR assay |
|------------------|---------------------|----------------------|---------------------|----------------------|
|                  | EC50 (μM) | CC50 (μM) | EC50 (μM) | CC50 (μM) | EC50 (μM) | CC50 (μM) | EC50 (μM) | CC50 (μM) |
| Teriflunomide    | 79.62     | >100     | 42.06     | >100     | >100     | >100     | >100     | >100     |
| Leflunomide      | 59.45     | >100     | 42.74     | >100     | >100     | >100     | >100     | >100     |
| Remdesivir (blinded) | 0.078     | >100     | <0.046    | >100     | <0.046   | 15.21    | 0.055    | 13.56    |
| Remdesivir (internal reference) | 0.071     | >100     | 0.015     | >100     | 0.032    | 14.95    | 0.070    | 13.37    |

References

[1] N. Zhua, D. Zhang, W. Wang, X. Li, B. Yang, J. Song, X. Zhao, B. Huang, W. Shi, R. Lu, P. Niu, F. Zhan, X. Ma, D. Wang, W. Xu, G. Wu, G.F. Gao, W. Tan, A novel coronavirus from patients with pneumonia in China, 2019, N. Engl. J. Med. 382 (2020) 727–733.

[2] A. Bar-Or, A. Pachner, F. Menguy-Vacheron, J. Kaplan, H. Wiendl, Teriflunomide and its mechanism of action in multiple sclerosis, Drugs 74 (2014) 659–674.

[3] D.A. Knight, A.Q. Hejmanowski, J.E. Dierskeheidt, J.W. Williams, A.S. Chong, W. J. Waldman, Inhibition of herpes simplex virus type 1 by the experimental immunosuppressive agent leflunomide, Transplantation 71 (2001) 170–174.

[4] A. Bilger, J. Plowsway, Sh. A. Ma, D. Navandan, E.A. Barlow, J.C. Romero-Masters, J. A. Brutströl, Z. Li, M.H. Tsai, H.J. Delecluse, S.C. Kenney, Leflunomide/teriflunomide inhibit Epstein-Barr virus (EBV)-induced lymphoproliferative disease and lytic viral replication, Oncotarget 8 (2017) 44266–44280.

[5] A. Liacini, M.E. Seemone, D.A. Munive, L.A. Tibbles, Anti-BK virus mechanisms of sirolimus and leflunomide alone and in combination: toward a new therapy for BK virus infection, Transplantation 90 (2010) 1450–1457.

[6] B.A. O’Hara, G.V. Gee, S.A. Haley, J. Morris-Love, C. Nyblade, C. Nieves, B. A. Hanson, X. Dang, T.J. Turner, J.M. Chavin, A. Lublin, I.J. Korainik, W. J. Atwood, Teriflunomide inhibits JCPyV infection and spread in glial cells and choroid plexus epithelial cells, Int. J. Mol. Sci. 22 (2021) 9809.

[7] Y. Enose-Akahata, N. Gngouth, J. Ohayon, M. Mandel, J. Chavin, T.J. Turner, S. Jacobson, Effect of teriflunomide on cells from patients with human T-cell lymphotropic virus type 1-associated neurologic disease, Neurol. Neuroimmunol. Neuroinflamm. 8 (2021) e986.

[8] M.C. Dunn, D.A. Knight, W.J. Waldman, Inhibition of respiratory syncytial virus in vitro and in vivo by the immunosuppressive agent leflunomide, Antivir. Ther. 16 (2011) 309–317.
[9] C.S. Sepúlveda, C.C. García, E.B. Damonte, Antiviral activity of A771726, the active metabolite of leflunomide, against Junin virus, J. Med. Virol. 90 (2018) 819–827.

[10] R. Xiong, L. Zhang, S. Li, Y. Sun, M. Ding, Y. Wang, Y. Zhao, Y. Wu, W. Shang, X. Jiang, J. Shan, Z. Shen, Y. Tong, L. Xu, Y. Chen, Y. Liu, G. Zou, D. Lavillette, Z. Zhao, R. Wang, L. Zhu, G. Xiao, K. Lan, H. Li, K. Xu, Novel and potent inhibitors targeting DHODH are broad-spectrum antivirals against RNA viruses including newly-emerged coronavirus SARS-CoV-2, Protein Cell 11 (2020) 723–739.

[11] A.M. Rabie, Teriflunomide: a possible effective drug for the comprehensive treatment of COVID-19, Curr. Res. Pharmacol. Drug Discov. 2 (2021), 100055.

[12] M. Wang, R. Cao, L. Zhang, X. Yang, J. Liu, M. Xu, Z. Shi, Z. Hu, W. Zhong, G. Xiao, Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro, Cell Res. 30 (2020) 269–271.

[13] B. Rozman, Clinical pharmacokinetics of leflunomide, Clin. Pharmacokinet. 41 (2002) 421–430.

[14] L. Prosperini, C. Tortorella, S. Haggiag, S. Ruggieri, S. Galgani, C. Gasperini, Determinants of COVID-19-related lethality in multiple sclerosis: a meta-regression of observational studies, J. Neurol. (2022) 1–11.

[15] Q. Wang, H. Guo, Y. Li, X. Jian, X. Hou, N. Zhang, J. Fei, D. Su, Z. Bian, Y. Zhang, Y. Hu, Y. Sun, X. Yu, Y. Li, B. Jiang, Y. Li, F. Qin, Y. Wu, Y. Gao, Z. Hu, Efficacy and safety of leflunomide for refractory COVID-19: a pilot study, Front. Pharmacol. 12 (2021), 581833.

[16] M. Dittmar, J.S. Lee, K. Whig, E. Segrist, M. Li, B. Kamalia, L. Castellana, K. Ayyanathan, P.L. Cardenas-Díaz, E.E. Morrissey, R. Traitt, W. Yang, K. Jurado, K. Samby, H. Ramage, D.C. Schultz, S. Cherry, Drug repurposing screens reveal cell-type-specific entry pathways and FDA-approved drugs active against SARS-CoV-2, Cell Rep. 35 (2021), 108959.