Prolyl isomerase Pin1 plays an essential role in SARS-CoV-2 proliferation, indicating its possibility as a novel therapeutic target

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Abstract

Novel coronavirus disease 2019 (COVID-19) has emerged as a global pandemic with far-reaching societal impact. Here we demonstrate that Pin1 is a key cellular molecule necessary for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) propagation. In this study, siRNA-mediated silencing of Pin1 expression markedly suppressed the proliferation of SARS-CoV-2 in VeroE6/TMPRSS2 cells. In addition, several recently generated Pin1 inhibitors showed strong inhibitory effects on SARS-CoV-2 proliferation, measured by both viral mRNA and protein synthesis, and alleviated the cytopathic effect (CPE) on VeroE6/TMPRSS2 cells. One compound, termed H-77, was found to block SARS-CoV-2 proliferation at an EC_{50} below 5 μM regardless of whether it was added to the culture medium prior to or after SARS-CoV-2 infection. The inhibition of viral N protein mRNA synthesis by H-77 implies that the molecular mechanism underlying SARS-CoV-2 inhibition is likely to be associated with viral gene transcription or earlier steps. Another Pin1 inhibitor, all-trans retinoic acid (ATRA)—a commercially available drug used to treat acute promyelocytic leukemia (APL) and which both activates the retinoic acid receptor and inhibits the activity of Pin1—similarly reduced the proliferation of SARS-CoV-2. Taken together, the results indicate that Pin1 inhibitors could serve as potential therapeutic agents for COVID-19.

Introduction

In December 2019, an outbreak of pneumonia occurred in Wuhan, China, caused by a virus later designated as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[1]. Following the outbreak, a global pandemic of SARS-CoV-2 infections has seriously disturbed daily life and economic activities, and intense efforts worldwide have been initiated to find effective therapies and vaccines to combat the pandemic. Notably, the lethality mortality rate of coronavirus disease 2019 (COVID-19) is higher in subjects with obesity, diabetes mellitus, and advanced age, in contrast to the high mortality rate in the young population observed in the case of the 1918 Spanish influenza pandemic. The prevailing hypothesis for this high lethality in a subset of individuals is that it is attributable to the more serious effects of a cytokine storm induced by SARS-CoV-2 in patients with chronic inflammatory status related to underlying obesity or diabetes mellitus.

Over the past several years, our research group has focused on elucidating the role of peptidyl-prolyl isomerase Pin1 in metabolic regulation[2]. There are three groups of peptidyl-prolyl isomerases (PPIases): the FKBP, Cyclophilin, and Parvulin families (Pin1 and Par14)[3]. Pin1 is unique among the PPIases in that it binds to pSer/pThr-Pro motifs and functions by modulating the enzymatic activity, protein stability, or subcellular localization of target proteins by catalyzing a cis-to-trans orientation of proline in its substrate's protein structure. Many studies have revealed roles of Pin1 in cancers, metabolism, and Alzheimer's disease. Evidence suggests that Pin1 expression in cancer cells is closely related to the degree of their malignancy, as Pin1 enhances cell proliferation and inhibits apoptosis. Nevertheless, Pin1 is not indispensable for the survival or growth of normal cells. Pin1 KO mice are born and become mature without any defects in size and appearance.

We have observed that Pin1 expression levels are markedly increased in several tissues including the liver, muscle, adipose tissue, and kidney in obese or diabetic mice[2] and in the livers of human subjects with hepatosteatosis (unpublished observation). Interestingly, Pin1 reportedly accelerates the proliferation of several viruses, although the molecular mechanism underlying Pin1-induced promotion of virus proliferation seems to differ among virus types. Taken together, we speculate that the increased Pin1 expression in obese or diabetic patients may be involved in the rapid progress and/or severity of infection with SARS-CoV-2.

Results

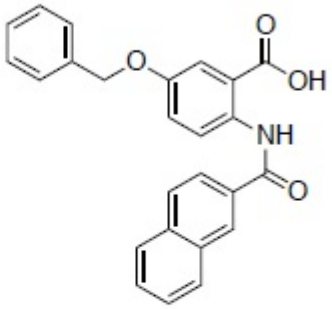
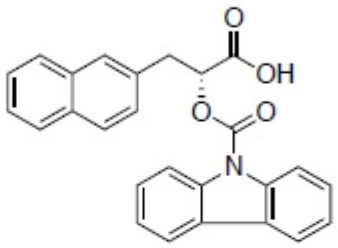
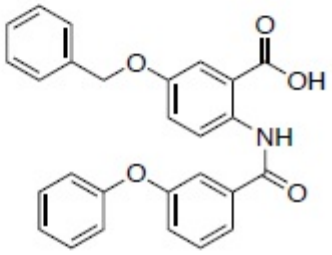
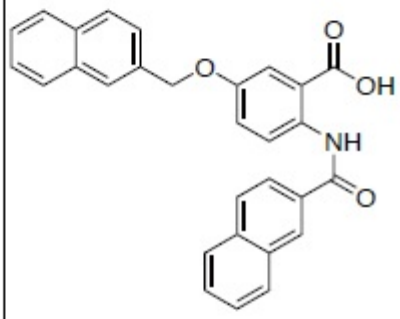
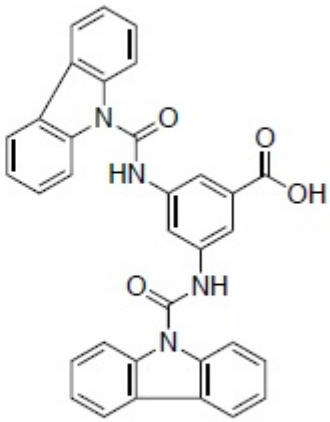
Essential role of Pin1 in SARS-CoV-2 proliferation

In this study, we examined the contribution of Pin1 to SARS-CoV-2 proliferation using VeroE6/TMPRSS2 cells, which are highly susceptible to SARS-CoV-2 infection due to their constitutive expression of transmembrane serine protease TMPRSS2[4]. Initially, we examined the effect of siRNA-mediated suppression of Pin1 expression on SARS-CoV-2 proliferation in VeroE6/TMPRSS2 cells. Treatment of the cells with either of two Pin1 siRNAs markedly reduced the expression of Pin1 protein and reduced the proliferation of SARS-CoV-2 in the cells as assessed by SARS-CoV-2 nucleocapsid protein levels detected in the cell lysates (Fig. 1A). Notably, the degree of reduction was more pronounced in our study than in a previous study in which feline coronavirus replication was partially suppressed by treatment with Pin1 siRNA[5]. Subsequently, we investigated the effect of Pin1 inhibitors on SARS-CoV-2 proliferation.

Inhibition of SARS-CoV-2 proliferation by Pin1 inhibitors

We have recently developed many novel compounds with Pin1 inhibitory activity, and they were experimentally characterized for their effects on SARS-CoV-2. Our experiments revealed that at least 20 of these compounds exhibit a strong suppressive effect on SARS-CoV-2 proliferation at a concentration of 10 μM. The chemical structures and the results for five representative compounds are shown in Table 1 and Fig. 1B, respectively. Studies on the viability of infected cells revealed that a cytopathic effect (CPE), syncytium formation, of VeroE6/TMPRSS2 cells by SARS-CoV-2 was also almost completely prevented by the addition of Pin1 inhibitors to the culture medium (data not shown). Among the inhibitors, compound H-77 almost completely blocked SARS-CoV-2 proliferation at a concentration of 5 μM. More detailed studies were therefore performed using this compound as a potent suppressor of SARS-CoV-2 proliferation. The concentration-dependent effect of H-77 against SARS-CoV-2 was shown by measuring viral protein levels in VeroE6/TMPRSS2 cells (Fig. 2A) or viral RNA isolated from the culture medium (Fig. 2B). Membrane fusion, a CPE caused by viral infection, became less apparent as the drug concentration was increased to 5 μM and was almost absent at concentrations above 7.5 μM (Fig. 2C). This trend was evident in the fusion index, which quantifies the degree of membrane fusion (Fig. 2D). Our studies revealed that 7.5 μM of H-77 substantially inhibited SARS-CoV-2 proliferation and its EC_{50} was estimated to be below 5 μM. Considering the data obtained by disrupting Pin1 activity with siRNA and various Pin1 inhibitory compounds, it can be concluded that Pin1 is essential for SARS-CoV-2 proliferation.

Table 1. Pin1 inihibitors that inhibited SARS-CoV-2 proliferation

H-77 [1)]		H-175 [2)]	
H-363 [1)]		H-371 [1)]	
H-596 [3)]			

1) T. Asano, Y. Nakatsu, H. Ito, T. Okabe, WO/2019/031472
2) T. Asano, Y. Nakatsu, H. Ito, T. Okabe, WO/2018/101329
3) T. Asano, Y. Nakatsu, H. Ito, T. Okabe, JP2020-191046

We therefore next investigated whether H-77 can exert its inhibitory effect even when added at the same time as the SARS-CoV-2 infection or after in order to determine its applicability as a therapeutic agent (Fig. 3A). Our results showed that H-77 almost completely blocked SARS-CoV-2 proliferation when added 2 h after infection and showed a weaker but still significant inhibitory effect when added 6 h after infection (Figs. 3B and *C*). The amount of genomic RNA released from the cells was significantly reduced by H-77 treatment (Figs. 2 and 3D). In addition, intracellular viral N mRNA was significantly reduced,

although some genome RNA was also mixed in (Fig. 3D), providing evidence that H-77 inhibits viral proliferation at the viral RNA transcription step or earlier.

Five potent Pin1 inhibitors, including H-77, were applied for 2 hours before virus infection, followed by washing out the Pin1 inhibitors before virus infection. N-protein synthesis of SARS-CoV-2 was strongly inhibited even after washout (Fig. 4A and *B*). These results indicate that the Pin inhibitor is effective if the cells are pretreated immediately before virus infection.

Inhibition of viral replication by a medical agent

At present, no highly specific Pin1 inhibitor is commercially available for either medical or experimental purposes. Although Juglone is the most commonly used Pin1 inhibitor compound for basic research, it reportedly binds to and inhibits the activity of many proteins, including tubulin, in addition to Pin1, and it was found that VeroE6/TMPRSS2 cells were unable to survive incubation with 2 μM Juglone for more than 12 h (data not shown). As an alternative, we tested all-trans retinoic acid (ATRA), an agonist of the retinoic acid receptor (RAR) that is used medically to treat acute promyelocytic leukemia (APL) and was recently reported to inactivate Pin1 isomerase activity[6]. The activities of ATRA as an RAR agonist and a Pin1 inhibitor both contribute to the suppression of APL cell growth[6]. As a result, it was found that ATRA similarly suppressed SARS-CoV-2 proliferation as shown by marked reductions in protein and viral RNA levels in a concentration-dependent manner (Fig. 5A and *B*) and alleviated its CPE (data not shown), although the EC_{50} of ATRA was higher than that of H-77.

Discussion

This study is the first study to demonstrate the essential role of Pin1 in SARS-CoV-2 proliferation and the possibility of Pin1 inhibition as a promising therapy against COVID-19. The inhibitory activities of our Pin1 inhibitors, H-77, H-175, H-363, H-371 and H-596, have been confirmed to inhibit Pin1 enzyme activity in an *in vitro* PPIase assay using recombinant Pin1 protein. However, potential non-specific effects on other proteins such as other PPIase enzymes or kinases have not yet been sufficiently ruled out. On the other hand, ATRA reportedly inhibits the activity Pin1 but does not affect the activity of FKBP or cyclophilin[6]. Thus, our results using Pin1 siRNAs and ATRA strongly support the involvement of Pin1 rather than that of other PPIases in the proliferation of SARS-CoV-2. We hypothesize that it is highly likely that the inhibitory effects of our five compounds on SARS-CoV-2 proliferation are mediated specifically through Pin1 inhibition, although the possibility of the existence of an additional mechanism(s) cannot be ruled out.

Interestingly, Pin1 has also been reported to enhance the proliferation of several other viruses including human immunodeficiency virus type 1 (HIV-1)[7], hepatitis C virus (HCV)[8], Epstein-Barr virus (EBV)[9], human T-lymphotropic virus type 1 (HTLV-1)[10], and feline coronavirus[5]. The molecular mechanisms underlying Pin1-induced enhancement of viral proliferation can be largely divided into two mechanisms. One is mediation by enhanced production of oncogenic or inflammatory proteins in the host cells via association of Pin1 with cyclin D1, NF-kB, and Tax[10]. The other is direct involvement of Pin1 in various

aspects of the life cycle of viruses such as core exuviation, genome integration, and RNA or DNA replication. For example, Pin1 has been shown to contribute to the uncoating of the HIV-1 core, reverse transcription of the RNA genome, and integration of HIV-1 genomic DNA into chromosomes[7]. In the case of EBV, Pin1 binds to the subunit of DNA polymerase, termed BALF5, and enhances replication[9]. Our results suggest that Pin1 plays a critical role in viral gene transcription or earlier steps after invasion of SARS-CoV-2 into cells and thus appears to be indispensable for SARS-CoV-2 proliferation. Further studies are necessary to identify the target protein of Pin1 and its functions in the life cycle of SARS-CoV-2.

In conclusion, our study clearly showed an essential role of Pin1 in SARS-CoV-2 proliferation. Accordingly, the use of Pin1 inhibitors might be an effective therapy against COVID-19. Our study also indicated the necessity for optimizing and/or developing novel compounds with both potent Pin1 inhibitory activity and high specificity.

Methods

Cell culture

VeroE6/TMPRSS2 cells (African green monkey kidney-derived cells expressing human TMPRSS2, purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank, JCRB1819) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1 mg/mL G418 at 37°C in 5% CO_2. For siRNA treatment, VeroE6/TMPRSS2 cells were transfected with either negative siRNA (QIAGEN) or Pin1 siRNA (Invitrogen) using RNAiMAX (Invitrogen) according to the manufacturer's protocol and subjected to SARS-CoV-2 infection 3 days later. Pin1 siRNA1: CCG UGU UCA CGG AUU CCG GCA UCC A. Pin1 siRNA2: GCC CUG GAG CUG AUC AAC GGC UAC A.

Pin1 inhibitors

Chemical structures of Pin1 inhibitors termed H-77, H-175, H-363, H-371 and H-593 are shown in Table 1. These Pin1 inhibitors inhibit isomerase activity by more than 80% at a concentration of 20 μM, based on an *in vitro assay* using recombinant Pin1 protein. However, it should be noted that the results of such an *in vitro* assay usually differ significantly from the results obtained by *in vivo* experiments. The compounds were solubilized in DMSO. Before the infection experiments, the culture medium of VeroE6/TMPRSS2 cells was changed to DMEM without FBS and G-418, and virus and/or Pin1 inhibitors were added at the indicated titer or concentrations.

SARS-CoV-2 infection

The SARS-CoV-2/JP/Hiroshima-46059T/2020 strain, which was isolated from a cluster infection in Hiroshima[11], was used. To prepare virus suspensions, VeroE6/TMPRSS2 cells were infected with the virus and incubated in DMEM. The virus titer was determined by the standard 50% tissue culture infectious dose ($TCID_{50}$) method and expressed as $TCID_{50}$/ml as described previously[12]. SARS-CoV-2 infection was performed in the BSL3 facility of Hiroshima University. Unless otherwise noted, VeroE6/TMPRSS2 cells were inoculated with SARS-CoV-2 at an input multiplicity of infection (MOI) of 0.01 for 24 h or an MOI of 10 for 8 h.

Membrane fusion and fusion index

Vero cells infected with SARS-CoV-2 at an MOI of 0.01 with or without a Pin1 inhibitor were observed with an inverted microscope and photographed with a microscope camera (INOCAM-HD2; Inohara, Hiroshima, Japan) on the following day. The cells were then washed with PBS, fixed with methanol, and stained with Giemsa staining solution. The fusion index was calculated as [1-(number of cells/number of nuclei)] as described previously[13]. Approximately 100 nuclei and cell number per field were counted, and the average fusion index of five fields was calculated.

Western blotting

Samples were prepared and electrophoresed on SDS-polyacrylamide gels, transferred to PVDF membranes, and subjected to immunoblotting using Supersignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA). The antibodies used were from GeneTex (Irvine, CA, USA) [SARS-CoV/SARS-CoV-2 (COVID-19) nucleocapsid antibody (GTX632269)] and Santa Cruz Biotechnology (Dallas, TX, USA) [β-actin (sc-47778) and Pin1 (sc-46660)].

Quantitative real-time PCR

Viral RNA was extracted from the collected samples from the culture medium using a NucleoSpin RNA Virus kit (MACHEREY-NAGEL GmbH & Co. KG., Düren, Germany) following the manufacturer's protocol. Cellular RNA in VeroE6/TMPRSS2 cells was prepared using the Maxwell RSC instrument (Promega Corp., Madison, WI) according to the manufacturer’s protocol. Conventional RT-qPCR for specific amplification of the nucleocapsid (N) gene of SARS-CoV-2 was performed using One Step PrimeScript III RT-qPCR mix (Takara Bio Inc., Kusatsu, Japan) according to the manufacturer's protocol. The Primer/Probe Set (2019-n) (Takara Bio Inc.) contains two primer sets, N and N2, both annealing to the N gene of SARS-CoV-2. The RNA genome quantity was determined using the new coronavirus positive control RNA (Nihon Gene Research Laboratory, Sendai, Japan) as a standard. Thermal cycling was carried out as follows: reverse transcription at 52°C for 5 min, initial denaturation at 95°C for 10 sec, 45 cycles of denaturation at 95°C for 5 sec, and a final annealing/extension at 60°C for 30 sec.

Declarations

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Author contributions

T.A. and T.S. conceived and designed the research. T.Y and Y.O performed the experiments using VeroE6/TMPRSS2 cells, and J.E., H.I. and T.O. produced and prepared the solution of Pin1 inhibitors. T.Y., T.S. and S.H. assisted with data analysis. T.A. and T.S. wrote the manuscript. All authors read and approved the manuscript.

Additional Information

The authors declare no competing interests.

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