Metformin Suppresses SARS-CoV-2 in Cell Culture

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Keywords

SARS-CoV-2, COVID-19, anti-viral activity, Metformin, AMPK, Diabetes

ABSTRACT

People with diabetes are reported to have a higher risk of experiencing severe COVID-19 complications. Metformin, a first-line medication for type 2 diabetes, has antiviral properties. Some studies have indicated its prognostic potential in COVID-19. Here, we report that metformin significantly inhibits SARS-CoV-2 growth in cell culture models. SARS-CoV-2 infection of gut epithelial cell line, Caco2, resulted in higher phosphorylation of AMPK. Metformin reduced viral titers in the infected cells by nearly 99%, and by about 90% when cells were treated prior to infection. Metformin pre-treatment resulted in further phosphorylation of AMPK and caused a ten-fold reduction of viral titers indicating its potential in preventing naïve infections. Confirming the positive impact of AMPK activation, another AMPK activator AICAR substantially inhibited of viral titers and, AMPK inhibitor Compound C, augmented it considerably. Metformin treatment post-SARS-CoV-2 infection resulted in nearly hundred-fold reduction of viral titers, indicating that the antiviral potency of the drug is far higher in infected cells, while still being able to reduce fresh infection. Metformin displayed SARS-CoV-2 TCID50 and TCID90 at 3.5 and 8.9 mM, respectively. In conclusion, our study demonstrates that metformin is very effective in limiting the replication of SARS-CoV-2 in cell culture and thus possibly could offer double benefits to diabetic COVID-19 patients by lowering both blood glucose levels and viral load.

INTRODUCTION

As the COVID-19 pandemic still rages on in most parts of the world, countries are racing to get their citizens fully vaccinated. While the vaccines are effective in most cases, emergence of newer variants of the causative agent, SARS-CoV-2, is a

cause for concern (Bernal et al., 2021). Particularly vulnerable to COVID-19 are patients with comorbidities such as cancer, auto-immune diseases, cardiovascular conditions, and diabetes. The hospitalization rate for patients with comorbidities who contracted COVID-19 was significantly higher during the first wave of the pandemic, associated with poor prognosis (Sanyaolu et al., 2020). Type 2 diabetes, one of the most common metabolic disorders, is universally treated using insulin and a number of other drugs, chiefly metformin (Davidson & Peters, 1997). Metformin is a biguanide compound, used as first-line antidiabetic medication worldwide. It acts primarily by increasing glucose intake and limiting gluconeogenesis in the liver, and its action is mediated in part by the energy-sensing kinase, 5'-AMP-activated protein kinase (AMPK) (Zhou et al., 2001). Metformin inhibits Complex I of the electron transport chain and suppresses ATP synthesis, which triggers AMPK activation. This results in a cascade of events that decreases anabolic processes and initiates macromolecular breakdown to reinstate homeostasis.

In the past year, a number of reports have debated the clinical use of metformin in COVID-19 (Bramante et al., 2021; Dardano & del Prato, 2021; Ibrahim et al., 2021; Zangiabadian et al., 2021). Many case studies on metformin treatment report a decrease in hospital mortality rates for patients that were on metformin prior to admission (Dardano & del Prato, 2021; L et al., 2020; Marmor et al., 2021). Reports suggest that high glucose levels are associated with poorer prognosis and well-controlled glucose levels were indicative of lesser complications (L et al., 2020). Metformin has also been reported to show anti-viral activity against other viruses (X. Chen et al., 2020). In this study, we aimed to investigate the effect of metformin on SARS-CoV-2, and identify the effects of AMPK perturbation on infection. Pre-treatment of cells with metformin prior to infection substantially lowered the viral titer.

Pharmacological activation of AMPK suppressed viral infection while its inhibition promoted it. Treatment of SARS-CoV-2 infected cells with metformin resulted in stronger restriction of the virus in a dose-dependent manner, as compared to the pre-treatment. Our results support the promising use of metformin as a therapeutic drug in COVID-19.

MATERIALS AND METHODS

Cell culture and reagents

Caco2, Huh7, and Vero cells were grown in DMEM supplemented with FBS, and Pen Strep, at 37°C and 5% CO_2. Anti-AMPKα antibody was procured from CST. GAPDH, β-tubulin, and anti-Nucleocapsid antibodies were from ThermoFisher Scientific. HRP-conjugated secondary antibodies were purchased from Jackson ImmunoResearch. Metformin, AICAR, and Compound C were procured from Merck Millipore.

Infections and treatments

All experiments involving virus culture were carried out in the biosafety level-3 laboratory at the Centre for Cellular and Molecular Biology (CCMB). SARS-CoV-2 strain B.1.1.8 (TG-CCMB-L1021/2020 isolate) was used for all experiments (Gupta et al., 2021) at 1 MOI. Cells were grown to 80% confluency and treated as described. For pre-treatments, cells were subjected to 10 mM metformin or 1 mM AICAR for 24 h, followed by infection with SARS-CoV-2 in serum-free medium (SFM) for 3 h in the presence of the respective compound. The inoculum was subsequently replaced with complete medium containing the compound and the cells were harvested at 24 h post-infection (hpi). In post-infection mode, the cells were first infected for 3 h after which the inoculum was replaced by media containing metformin and further incubated until 24 hpi. Dose-dependent effect of metformin

was studied by subjecting cells to varying doses of metformin (5, 10, 20, and 40 mM) similar to the post-treatment regimen mentioned above. Compound C treatment was carried out by infecting cells for 3 h at 1 MOI, complete media for 21 h, and then an additional 24 h with 10 µM Compound C. For all treatments, media supernatant was collected to measure extracellular viral RNA as well as infectious viral titres, and cells were processed for immunoblotting.

Virus quantification and titration

RNA from viral supernatants was isolated using Nucleospin Viral RNA isolation kit (Macherey-Nagel GmbH & Co. KG). qRT-PCR was carried out using nCOV-19 RT-PCR detection kit from Q-line Molecular to quantify SARS-CoV-2 RNA following manufacturer's protocol on Roche LightCycler 480.

Infectious titres of the supernatants were calculated using plaque forming assay (PFU/mL) as mentioned previously (Gupta et al., 2021). Briefly, the supernatant was serially diluted from 10^{-1} to 10^{-7} in SFM and added to a confluent monolayer of Vero cells for infection for 3 h. The medium was then replaced with a 1:1 mixture of agarose: 2 × DMEM (1% low-melting agarose (LMA) containing a final concentration of 5% FBS and 1 × Pen-Strep). Six days post-infection, cells were fixed in 4% formaldehyde prepared in 1× PBS and subsequently washed and stained with 0.1% crystal violet to count the plaques.

Immunoblotting

Protein pellets were lysed in an NP-40 lysis buffer as described earlier (Gupta et al., 2021). Protein quantification was done using BCA method (G Biosciences). Lysates were then mixed with 6 × Laemmli buffer, and equal amounts of protein were run on SDS-PAGE, followed by transfer onto PVDF membrane. Blots were blocked in 5%

BSA and incubated with specific primary antibodies at 4°C overnight. Incubation with HRP-conjugated secondary antibodies was done for 1 hour and the blots were developed on a BioRad Chemidoc MP system using ECL reagents (ThermoFisher and G Biosciences). Quantification was performed using ImageJ (Schneider et al., 2012).

Cell viability assay

Effect of different doses of metformin on viability of mock and infected Caco2 cells was measured using MTT assay. Cells were subjected to varied doses of metformin as mentioned in the previous section. After incubation, media containing 0.5 mg/mL MTT was added to cells and incubated at 37°C for 3.5 h. Formazan crystals were dissolved in 100 µL DMSO and incubated for 30 min with mild agitation. Viability was read as absorbance measured at 570 nm, with a reference reading at 620 nm.

Statistical analysis

All experiments were performed in triplicate to calculate mean ± SEM. Statistical significance was calculated using two-tailed, unpaired Student's *t*-test and *p* values are represented as *, **, ***, indicating $p \leq 0.05$, 0.005, and 0.0005, respectively. IC50 and IC90 were calculated from qRT-PCR data, while TCID50 and TCID90 were calculated from PFU data from metformin titration experiments.

RESULTS

SARS-CoV-2 infection causes long-term phosphorylation of AMPK

Caco2 cells were infected with 1 MOI of SARS-CoV-2 for several time points and the samples were analyzed for AMPK phosphorylation. Though no significant change in the AMPK phosphorylation was detected until 48 h post-infection (hpi) (Figure 1 A and B), marked increase in phosphorylation was evident from 48 hpi that further

strengthened until 96 hpi, despite a drop in the abundance of the protein. These results indicated a major metabolic reprogramming, resulting in AMPK phosphorylation occurring after 24 hpi. Interestingly, AMPK phosphorylation coincided with the accumulation of viral proteins (Figure 1A).

Metformin protects cells from SARS-CoV-2 infection

We investigated the role of AMPK activation during SARS-CoV-2 infection as previous reports have clearly established the roles played by this molecule on the outcome of viral infections (Bhutta et al., 2021). Caco2 cells were pre-treated with 10mM concentration of metformin for 24 h after which they were infected with 1 MOI of SARS-CoV-2 for 3 h in presence of metformin. Subsequently, the viral media was replaced with growth medium containing metformin and incubated until 24 hpi (Figure 1C). Increased phosphorylation of AMPK in the drug-treated cells confirmed the effect of metformin (Figures 1 D and E). Metformin treatment resulted in nearly 50% drop in the viral RNA (Figure 1F), and nearly one-log drop in the infectious viral titers (Figure 1G) indicating that metformin treatment is protective against SARS-CoV-2 infection. However, no prominent change in the viral protein N was observed (Figures 1 D and H), suggesting that viral translation or its stability is not negatively impacted by metformin. Metformin treatment in Huh7 cells also brought about significant reduction in the infectious viral titer of SARS-CoV-2 (Supplementary Figures S1 A-D), albeit, less pronounced than in Caco2 cells, confirming that metformin has strong protective effects against SARS-CoV-2 infection.

AMPK activation restricts SARS-CoV-2

To further verify if the protective effect of metformin involves AMPK, we used AICAR, another activator of AMPK. Cells were pre-treated with 1mM of AICAR for 24 h as in

the case of metformin (Figure 2A). AICAR treatment (Figure 2B) significantly lowered the infectious viral titer of SARS-CoV-2 (Figure 2C) by almost one-log, as did metformin. These results demonstrate that AMPK activation is certainly beneficial to the host cells by significantly limiting the viral titers. We further confirmed this effect by inhibiting AMPK by using Compound C (CC) during SARS-CoV-2 infection. Since AMPK phosphorylation peaked beyond 24 h, cells were first infected at 1 MOI for 3 h followed by supplementation with growth medium. 24 hpi, media containing 10 µM CC was added and incubated for an additional 24 h (Figure 2D). Though CC treatment caused a visible drop in AMPK phosphorylation in mock cells, there was no apparent decrease observed in infected cells, indicating that the virus-induced AMPK activation overrides CC inhibition (Figure 2 E and F). As anticipated, CC treatment resulted in over four-fold higher viral titers in the supernatants as against the control sample (Figure 2G), accompanied by a modest drop in N levels (Figure 2H). CC treatment of infected Huh7 cells also resulted in substantial increase in the infectious viral titres (Supplementary Figures S2 A, B, and D), again accompanied by considerable drop in N levels (Supplementary Figure S2C). These results confirm that AMPK coordinates strong antiviral measures in SARS-CoV-2 infected cells. Thus, activation of AMPK during the infection is protective against SARS-CoV-2 infection.

Metformin treatment post-infection causes more profound restriction of SARS-CoV-2

We next tested the effect of metformin in Caco2 cells previously infected with SARS-CoV-2 to extrapolate its impact on the infected patients. Cells infected with 1 MOI of SARS-CoV-2 for 3 h were subsequently treated with 10 mM metformin until harvested at 24 hpi (Figure 3 A). Metformin caused higher AMPK phosphorylation

(Figures 3 B and C). In comparison with the pre-treatment regimen, the post-infection regimen caused a more profound drop in the viral RNA and nearly two log decrease of infectious titer in the supernatant (Figures 3 D and E, respectively). Interestingly, considerable drop in the levels of N was observed in the metformin treated samples (Figure 3F), further confirming the profound restrictive effect of the drug on SARS-CoV-2. In comparison, N levels were relatively unchanged in the samples pre- and co-treated with metformin and AICAR (Figures 1 D, H and 2B respectively). A higher drop in the viral titers were observed in metformin treated Huh7 cells as well (Supplementary Figures S3 A and B). These results indicate that the protection offered by metformin from SARS-CoV-2 infection is more profound in cells previously infected with SARS-CoV-2 than the uninfected cells.

Since post-infection regimen had a higher impact on SARS-CoV-2, we performed a dose-dependence analysis. A dose-dependent increase in AMPK phosphorylation was evident in SARS-CoV-2 infected cells from 5-40 mM metformin concentrations (Figures 4 A and B). A gradual and dose-dependent decrease in N levels was evident (Figures 4 A and C). The drop in viral RNA levels was more dramatic with a very significant drop detected even at 5 mM concentration and was further stabilized at 20 mM concentration (Figure 4D). 5 mM concentration of metformin inhibited infectious viral titers by 70% while the highest inhibition of nearly 2 logs was observed at 20 mM concentration where once again, the inhibition was stabilized (Figure 4E). The calculated IC50 and IC90 for viral RNA were measured to be 2.9 mM and 8.8 mM respectively (Figure 4F). We also measured the TCID50 and TCID90 values at 3.5 mM and 8.9 mM respectively (Figure 4G). MTT experiments carried out with the different doses indicate a steady decrease in viability with

increasing metformin (Figure 4H). Together, these results unambiguously demonstrate a potent anti-SARS-CoV-2 effect of metformin.

DISCUSSION

The anti-diabetic drug, metformin, has been projected to influence the prognosis of COVID-19 patients. As patients with comorbidities fared worse when infected with SARS-CoV-2, management of an ongoing illness alongside COVID-19 treatment became paramount. Some studies early during the pandemic identified a positive correlation between improved glucose levels in diabetic patients on metformin and better clinical outcome (Y. Chen et al., 2020; Crouse et al., 2020; L et al., 2020; P et al., 2020). A number of reports proposed metformin as a possible “miracle or menace” in COVID-afflicted patients, based on retrospective data from hospitalisations, (Lui & Tan, 2021; Marmor et al., 2021) comparing the length of hospitalisation, severity of symptoms, or mortality. In this study, we demonstrated that metformin profoundly lowers SARS-CoV-2 infectivity. While extrapolating these results to a clinical set up may not be appropriate, our data indicate that metformin can be effective not only as a treatment option, but as a prophylactic agent as well. With this data, we suggest that treatment of patients with metformin prior to infection with SARS-CoV-2 may have assisted in decreasing their symptoms of COVID-19. Our results also suggest that metformin could be beneficial in non-diabetic, COVID-19 patients and expand the scope of its coverage. In summary, this data lies in agreement with the numerous case studies published during the pandemic that suggested an antiviral role for this known anti-diabetic drug.

Metformin plays a major role in modulating lipid metabolism, but the mechanisms are multi-dimensional. Metformin is a soluble compound that interferes with Complex I

of the electron transport chain, and the decrease in ATP production causes AMPK activation. It also decreases hepatic lipids, increases skeletal muscle uptake of glucose, and in parallel helps in decreasing circulating lipids that can eventually increase cardiovascular risk especially in diabetic adults who are obese (Pernicova & Korbonits, 2014; Rena et al., 2017). RNA viruses have an intimate relationship with the cytoplasmic membrane network and modulate lipogenesis to steer cells to produce more vesicles to aid replication, as well as packaging and release (Herker & Ott, 2012; Pereira-Dutra et al., 2019). The impairment of such activities leading to loss in infectivity has been reported for several viruses (Abu-Farha et al., 2020; X. Chen et al., 2020; CN et al., 2021). A recent report on SARS-CoV-2 highlighted the possible role that lipid droplets play in its infection (Dias et al., 2020). Not only did they observe higher colocalization of viral RNA with the lipid droplets, they also demonstrated that inhibition of its formation decreased viral load as well as pro-inflammatory cytokines and apoptosis markers. These results in conjunction with ours indicates that the anti-viral effect of metformin is probably a resultant of altered lipid metabolism.

We speculate that the loss in infectivity of SARS-CoV-2 by metformin could also be, an outcome of altered lipid metabolism mediated by AMPK. AMPK affects cellular lipid levels through a number of its substrates, such as ACC and SREBP1. AMPK also regulates macromolecular metabolism, mitochondrial homeostasis, autophagy as well as apoptosis. As its role is multifaceted and vital for maintaining energy levels, it has been reported to play key roles in many virus infections. Multiple reports shows that AMPK activation can be either detrimental or beneficial for virus survival and propagation (Bhutta et al., 2021). Our results using AICAR and CC in SARS-CoV-2 infection implies an unfavorable/antiviral environment for the virus when

AMPK is activated. In this context, it is interesting to note that N protein was detected at modestly higher abundance during pharmacological activation of AMPK unlike the viral RNA and infectious titer, indicating that viral protein translation is not inhibited during the treatments. However, a significant drop in N levels during the post-infection treatment suggested an overall inhibition of viral life-stages concurrent with an overall drop of cellular activities indicated by MTT results. Inhibition of metabolic activities particularly in the infected cells upon post-infection metformin treatment indicated that metformin treatment specifically targeted the infected cells for destruction. This could be viewed as beneficial to the system fighting to clear the virus from it.

Although multiple reports show AMPK as the major effector of metformin action, it is now well established that metformin exerts its affects through other pathways such as PKA and FBPase-1 mediated regulation as well (Pernicova & Korbonits, 2014). Further study into the mechanism SARS-CoV-2 inhibition by metformin could pave the way for it to be a possible therapeutic target for COVID-patients. From a clinical perspective, our study provides some answers to the favorable prognosis of metformin-treated diabetic patients who contracted COVID-19.

Institutional biosafety

Institutional biosafety clearance was obtained for the experiments pertaining to SARS-CoV-2.

Author contributions

H.P. performed treatments, infections, quantification and immunoblotting. D.N. performed qRT-PCR experiments. H.P. and K.H.H. conceptualized the study and wrote the manuscript.

Acknowledgement

We thank Divya Gupta, Vishal Sah, Sai Poojitha, and Prangya Paramita Sahoo for their help with generation of virus and for conducting experiments. We specially thank Mohan Singh Moodu and Amit Kumar for their assistance with logistics.

Funding

The work was supported by the internal funding from CSIR-CCMB.

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LEGENDS

Figure 1. SARS-COV-2 infection induces AMPK phosphorylation and metformin treatment inhibits SARS-CoV-2. (A) Immunoblots analyzing the phosphorylation of AMPK in SARS-CoV-2 infected Caco2 cells for early (1, 2, 6, and 12 h) and late (24, 48, 72, and 96 h) time points. Cells infected with 1 MOI of SARS-CoV-2 were harvested at various time intervals post-infection and analyzed by immunoblotting. **(B)** Quantitative representation of AMPK phosphorylation from three independent replicates. Densitometric values of p-AMPK bands were normalized against those of T-AMPK and GAPDH belonging to the corresponding samples and the values were plotted graphically. **(C-H)** Pre-treatment with metformin suppresses

SARS-CoV-2 infection in Caco2 cells. **(C)** Schematic of the experimental set up for pre-treatment. Cells were infected with SARS-CoV-2 24 h after metformin treatment. The vehicle control cells were also infected with the virus in parallel. **(D)** Immunoblots confirming AMPK phosphorylation by metformin or vehicle treatment and SARS-CoV-2 infection. **(E)** Densitometric quantification of AMPK phosphorylation in infected cells. **(F)** SARS-CoV-2 RNA levels in the supernatant of metformin treated samples, measured by qRT-PCR of E gene. Relative fold change in the E levels between metformin and vehicle treated samples is depicted. **(G)** Relative fold change in the infectious viral titers of SARS-CoV-2 in metformin treated samples compared against that treated with vehicle, represented as fold change in PFU/mL. **(H)** Densitometric analysis of N expression during metformin treatment.

Figure 2. AMPK activation is beneficial to the host against SARS-CoV-2. (A) Schematic of the treatment of Caco2 cells with AICAR and infection by SARS-CoV-2. **(B)** Immunoblot confirming the infection. **(C)** Relative infectious titers of SARS-CoV-2 in samples that underwent pre-treatment with AICAR, as against the vehicle. **(D)** Schematic of the treatment of SARS-CoV-2 infected Caco2 cells with CC. **(E)** Immunoblot confirmation of the infection and inhibition of AMPK activity. **(F)** Quantification of AMPK phosphorylation in CC treated, infected samples. **(G)** Relative infectious titers of SARS-CoV-2 in samples that underwent CC-treatment, as against the vehicle, DMSO. **(H)** Relative expression of N in SARS-CoV-2 infected cells treated with CC or DMSO, quantified from densitometric values.

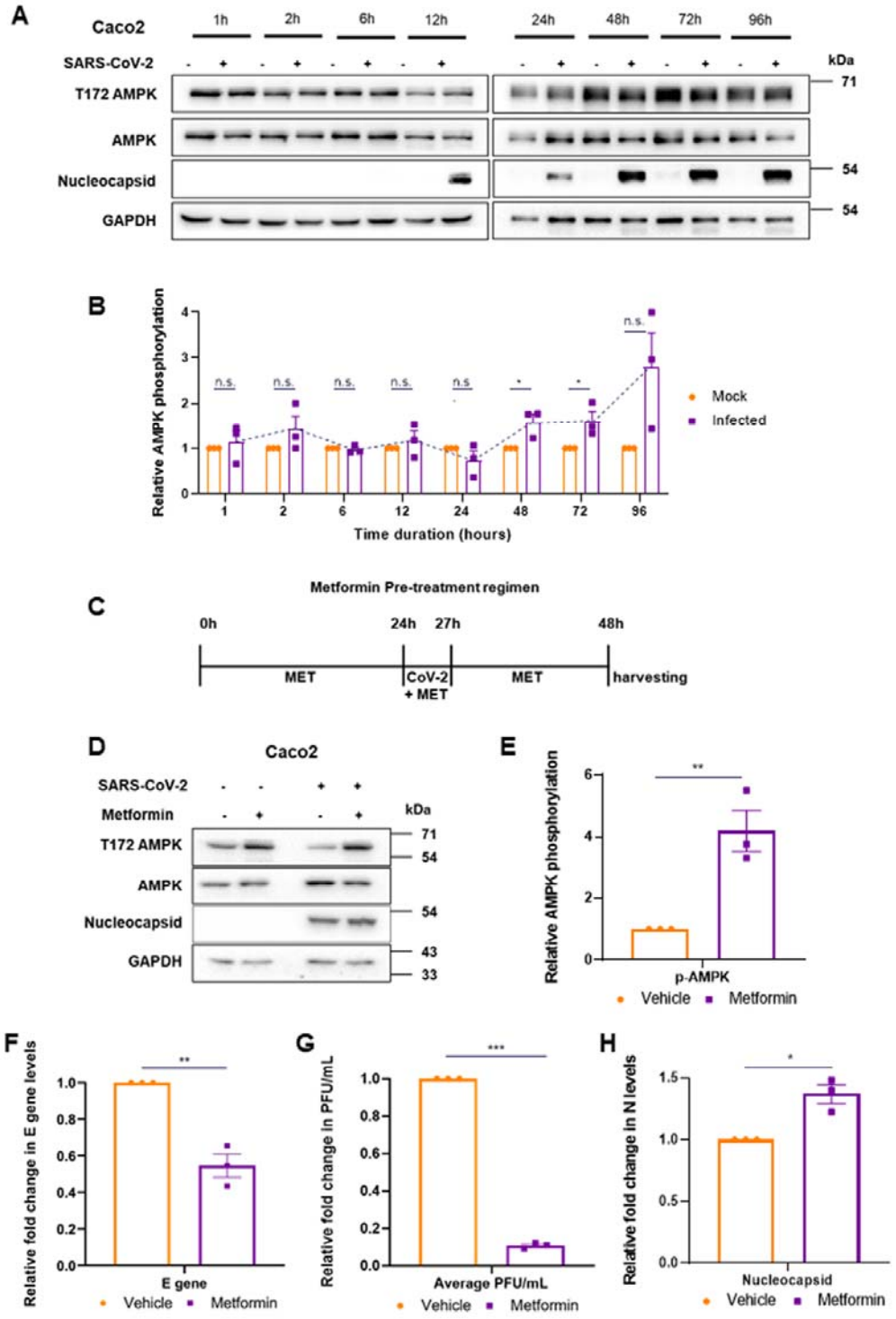
Figure 3. Metformin suppresses SARS-CoV-2 replication in the infected cells. (A) Schematic of the post-infection treatment by metformin. **(B)** Confirmation of SARS-CoV-2 infection and AMPK phosphorylation by immunoblotting. **(C)** Densitometric analysis of AMPK phosphorylation in the treated, infected cells. **(D)**

Relative fold change in SARS-CoV-2 E gene measured by qRT-PCR in the supernatants of metformin treated cells compared with the those treated with vehicle. **(E)** Relative SARS-CoV-2 infectious titers of the supernatant from samples treated with metformin represented as fold change in PFU/mL. **(F)** Relative abundance of N levels in the metformin treated samples against the control.

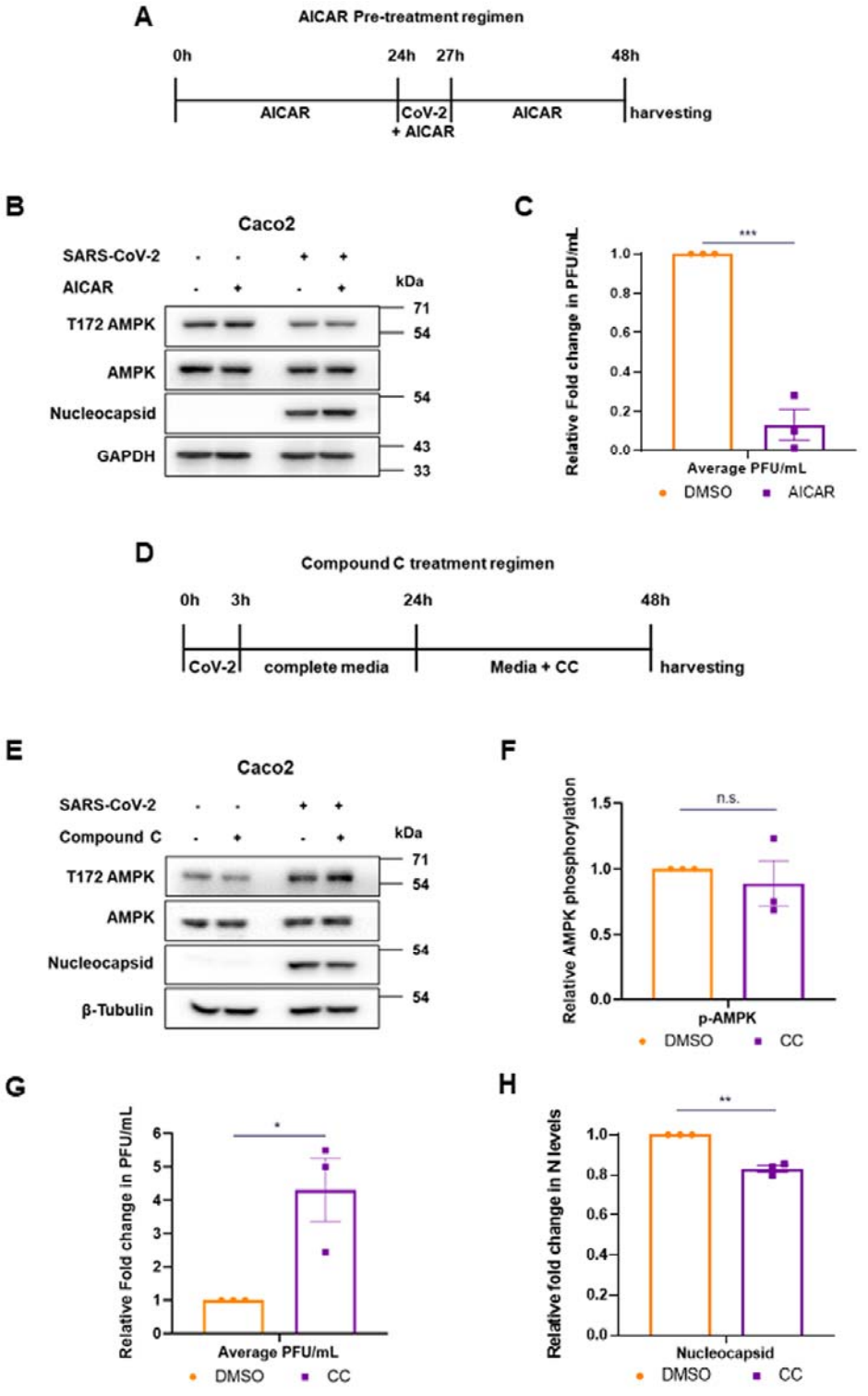
Figure 4. Metformin treatment inhibits SARS-CoV-2 replication in a dose-dependent manner. (A) Immunoblots confirming the infection and AMPK phosphorylation following the treatment of SARS-CoV-2 infected cells with metformin at the doses described above the panel. **(B)** Relative AMPK phosphorylation in the samples treated with metformin. The graph was generated from the densitometric analysis of the immunoblots. **(C)** Relative fold change in SARS-CoV-2 E gene measured by qRT-PCR in the supernatants of metformin treated cells compared with the those treated with vehicle. **(D)** Relative SARS-CoV-2 infectious titers of the supernatant from samples treated with metformin represented as fold change in PFU/mL. **(E)** Relative abundance of N quantified from the immunoblots from the panel (A). **(F)** Calculation of IC50 and IC90 for metformin on SARS-CoV-2 replication measured by plotting E gene levels present in the supernatants of the samples treated with the respective concentrations of metformin. **(G)** Measurement of TCID50 and TCID90 for metformin on SARS-CoV-2 infectious virus particle production. PFU/mL data for the individual samples treated with the specific concentrations were plotted in the graph to calculate the respective values. **(H)** Analysis of cell viability in the metformin-treated or the control cells by MTT assay. % viability of cells from absorbance measurements at 570 and 620 nm relative to the respective vehicle control are plotted graphically.

FIGURES

Figure 1



443 **Figure 2**



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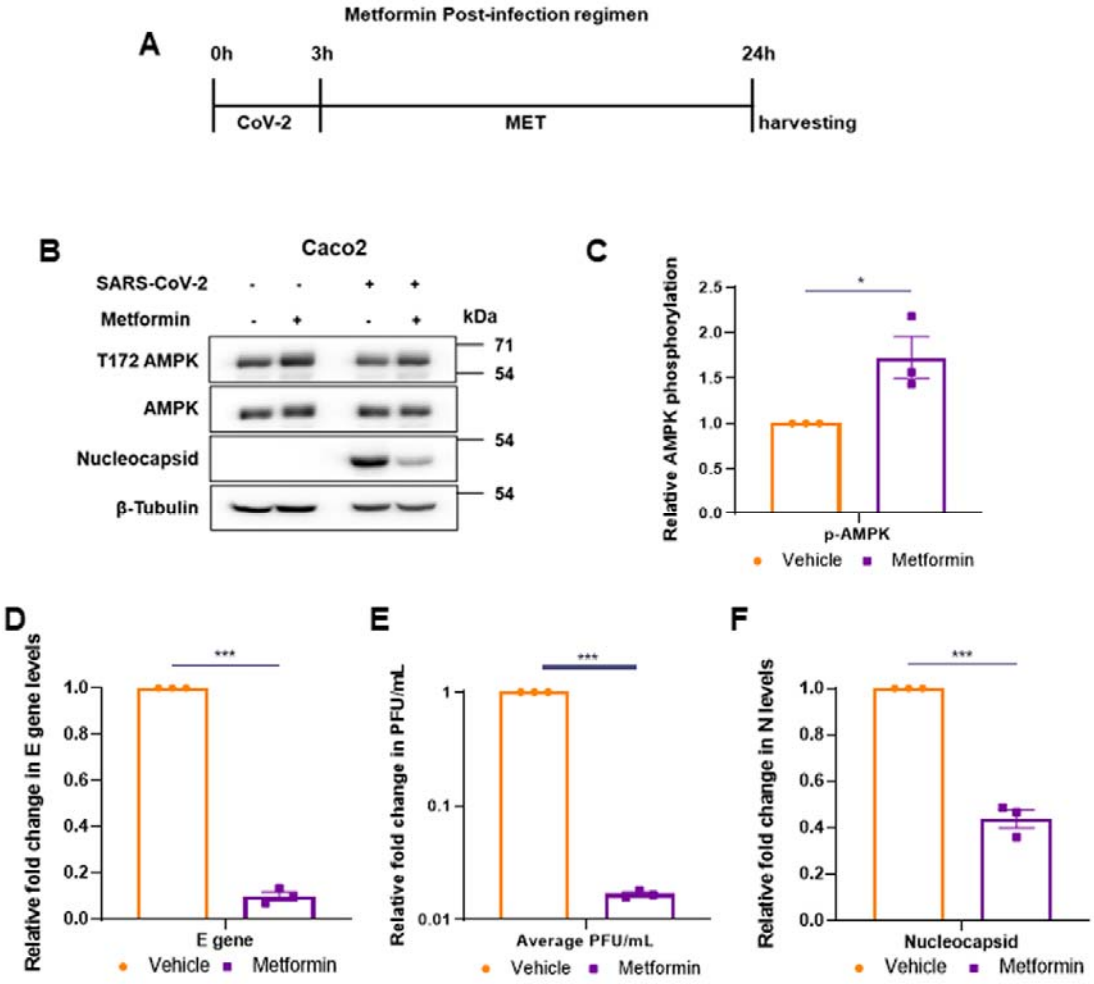
445 **Figure 3**



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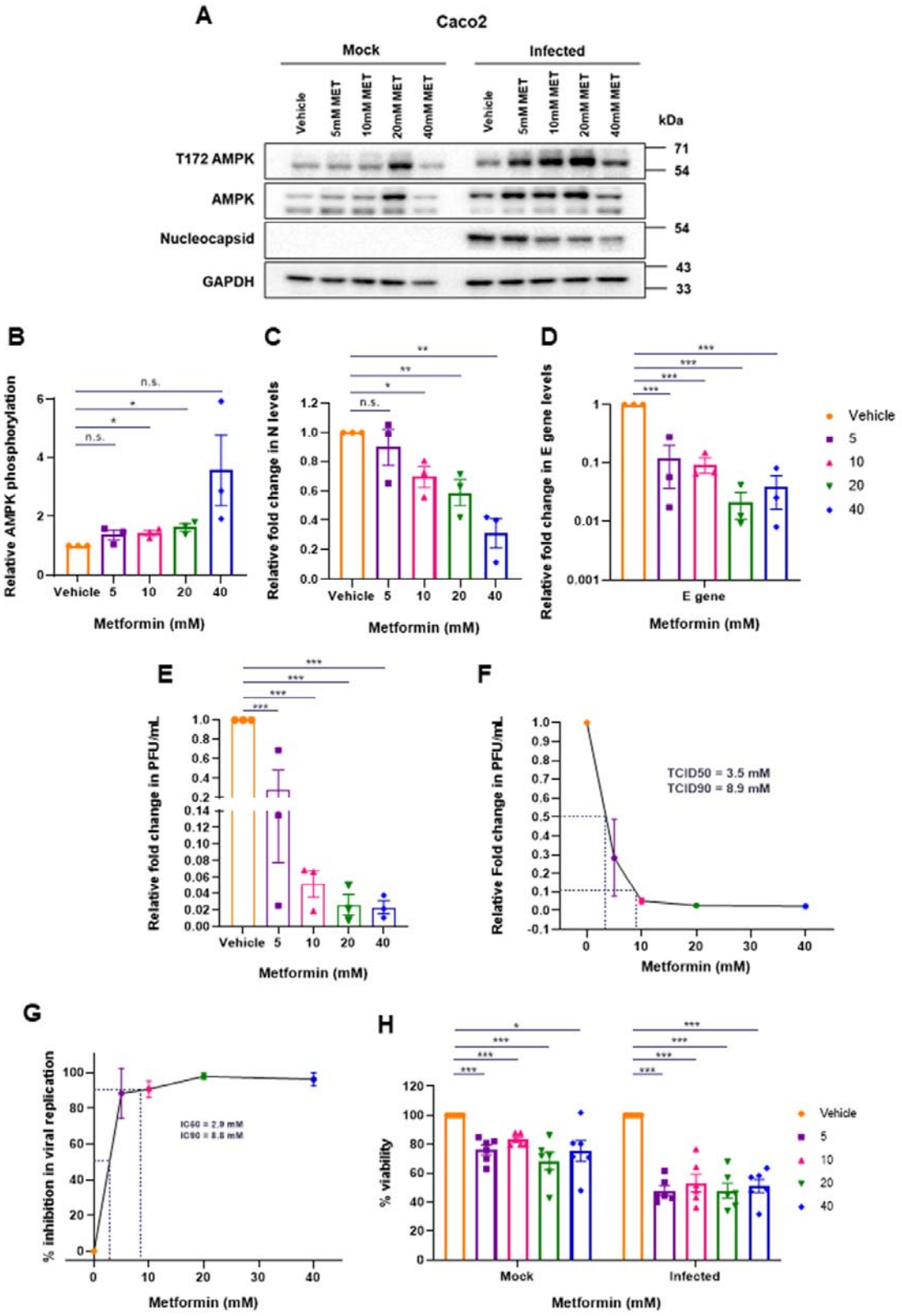
452 **Figure 4**



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Supplementary figures

Figure S1

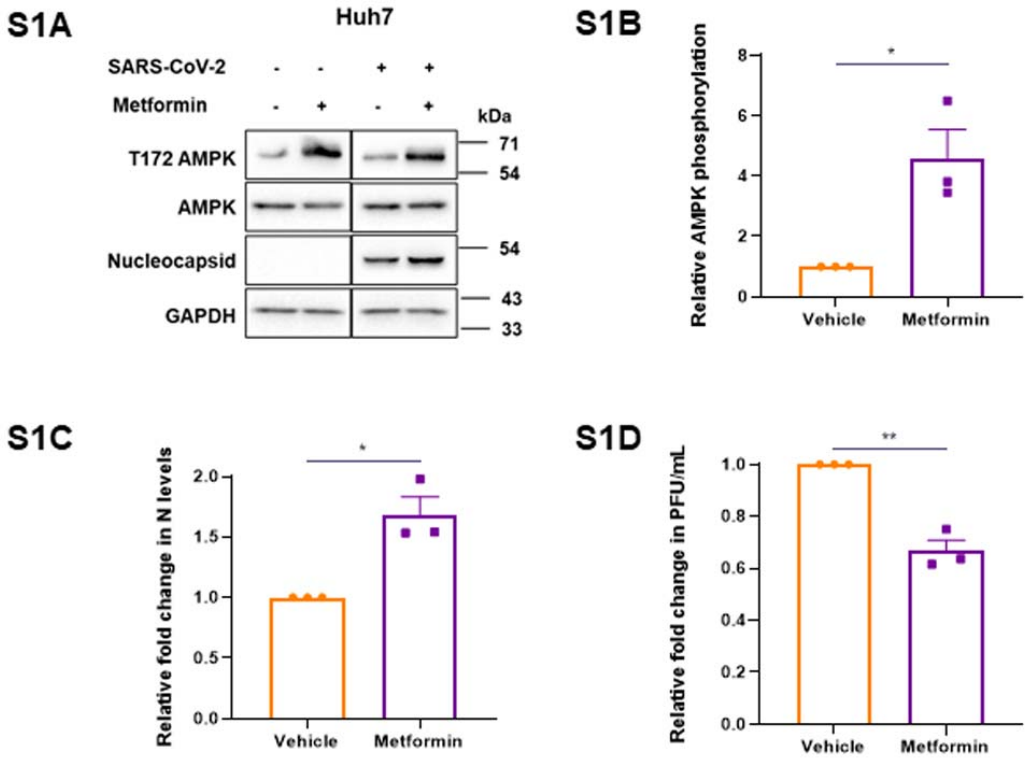


Figure S1. Metformin treatment reduces SARS-CoV-2 infection in Huh7 cells. **(A)** Immunobloting of the metformin treated or control samples to confirm SARS-CoV-2 infection an AMPK phosphorylation. Huh7 cells were treated with metformin and infected with SARS-CoV-2 as demonstrated in Figure 1D and the samples processed as in Figure 1E. **(B)** Densitometric quantification of AMPK phosphorylation and **(C)** that of N abundance in metformin treated or control samples. **(D)** Relative infectious titer of SARS-CoV-2 in the supernatants of metformin treated samples or the control samples.

Figure S2

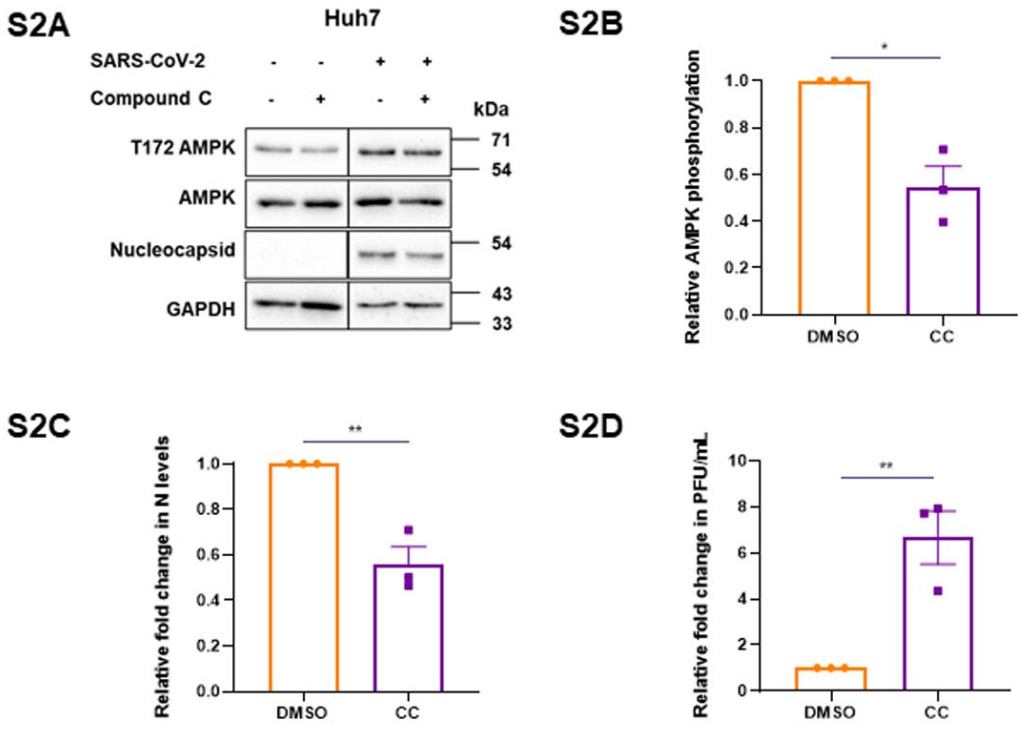
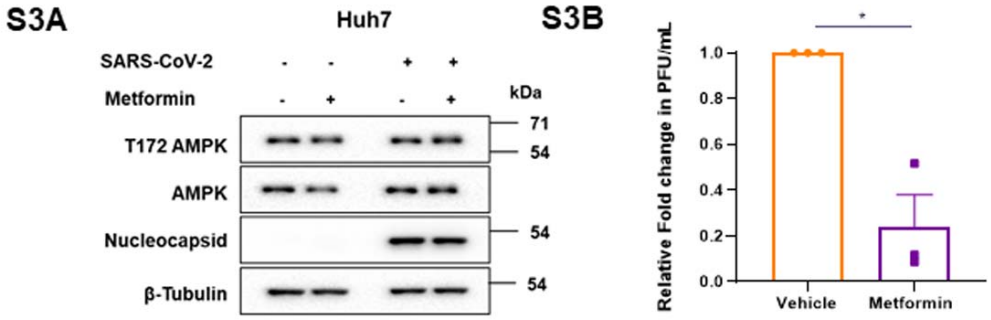


Figure S2. AMPK inhibition promotes SARS-CoV-2 titers. **(A)** Confirmation of SARS-CoV-2 infection in Huh7 cells by immunoblotting. **(B)** Relative AMPK phosphorylation levels in samples treated with CC or the control samples. **(C)** Relative abundance of N in the control samples or those treated with CC. **(D)** Relative infectious titers in the control or CC-treated samples represented as relative PFU/mL.

478 **Figure S3**



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480 **Figure S3.** Metformin inhibits SARS-Cov-2 replication in the infected Huh7 cells. **(A)**
481 Immunobloting of SARS-Cov-2 infected Huh7 cultures treated with metformin
482 compared with the control samples. The cells were first infected with the virus and
483 followed by metformin treatment as described in Figure 3A. **(B)** Relative infectious
484 titer of SARS-Cov-2 from the control samples or those treated with metformin.