Inhibiting cytomegalovirus replication through targeting the host electron transport chain

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Abstract

Human cytomegalovirus (HCMV) is a near ubiquitous herpesvirus that relies on host cell metabolism for efficient replication. Although it has been shown that HCMV requires functional host cell mitochondria for efficient replication, it is unknown whether mitochondrial targeted pharmacological agents can be repurposed as antivirals. Here we report that treatment with drugs targeting the electron transport chain (ETC) complexes inhibit HCMV replication. Addition of rotenone, oligomycin, antimycin and metformin resulted in decreased HCMV titers in vitro, independent of HCMV strain. This further illustrates the dependence of HCMV replication on functional mitochondria. Metformin, an FDA approved drug, delays HCMV replication kinetics resulting in a reduction of viral titers. Repurposing metformin as an antiviral is advantageous as its safety profile and epidemiological data are well accepted. Our findings provide new insight into the potential for targeting HCMV infection through host cell metabolism and how these pharmacological interventions function.

Keywords

Cytomegalovirus; Metformin; Electron transport chain; Mitochondria; CMV

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Declaration of competing interest

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1. Introduction

Human cytomegalovirus (HCMV) is a large DNA herpesvirus that may reach 85% seroprevalence rates in some populations (Cannon et al., 2010). In the US, there are ~40,000 congenitally acquired HCMV infections per year making it the leading cause of birth defects related to infectious disease. HCMV can also cause severe direct and indirect pathologies under immunosuppressed conditions (Alford et al., 1990; Adler, 2005). Despite monitoring and antiviral therapies as standard operating procedure, HCMV infection and/or reactivation remains a critical obstacle to successful transplantation due to severe pathologies, graft loss or even death (Razonable et al., 2013; Ljungman et al., 2011; Fuji et al., 2017; Yadav et al., 2017). The need for improved antivirals to treat HCMV is highlighted by the absence of an effective vaccine, ineffective neutralizing antibody therapies and limited efficacy of HCMV antivirals (Lurain and Chou, 2010; Fouts et al., 2014). The viral DNA synthesis inhibitors ganciclovir, valganciclovir, foscarnet and cidofovir are the antivirals of choice but are all limited due to resistant HCMV strains and therapeutic compatibility (Dropulic and Cohen, 2010). To illustrate these further, clinical isolates of HCMV have already been reported to be resistant to letermovir (Prevymis®) a recently approved antiviral (FDA approval 11/08/2017) for the prophylaxis of HCMV infection during post-transplantation (Chemaly et al., 2014; Lischka et al., 2016; Bowman et al., 2017; Goldner et al., 2014; Jung et al., 2019).

Studies examining changes to metabolism after HCMV infection have led to a renewed interest in viral-host cell metabolic reprogramming (Munger et al., 2006, 2008; DeVito et al., 2014; Chambers et al., 2010; Yu et al., 2011, 2014; Xi et al., 2019). Data suggests that HCMV alters host cell metabolic pathways to meet the biomass and bioenergetic demands of viral replication. We recently showed that host mitochondrial function and morphology is altered by HCMV to meet the metabolic demands of viral replication (Combs et al., 2019). Mitochondria are the powerhouse of the cell but are integral in numerous other cellular functions. Signal transduction, metabolism, immune response, cell cycle, and apoptosis are all associated with mitochondrial function (Antico Arciuch et al., 2012; Chandel, 2014). Through utilization of glucose, amino acids and fatty acids, mitochondria produce ATP, maintain redox balance and provide biosynthetic precursors. All of these mitochondrial-associated pathways are exploited by HCMV during replication (Munger et al., 2008; Chambers et al., 2010; Yu et al., 2011; McArdle et al., 2011; Spencer et al., 2011; Karniely et al., 2016; Monk and Zwezdaryk, 2020).

In this study, we evaluated the potential of targeting the host cell mitochondrial electron transport chain (ETC) as an antiviral approach. HCMV infected cells were treated with pharmacological agents specific to ETC complex I, III or ATP synthase. We also tested the antiviral efficacy of metformin, an FDA approved biguanide class drug used for the treatment of type II diabetes. Metformin is widely prescribed clinically, exhibits a strong safety profile, reproducibly reduces glucose levels under hyperglycemic conditions and is being evaluated as a possible anti-tumor agent and promoter of healthy aging (Camacho et al., 2015; Cabreiro et al., 2013; Knowler et al., 2002). The precise mechanism of action for metformin is still unknown, but it is believed to disrupt mitochondrial function by altering efficient transport of electrons contained in nicotinamide adenine dinucleotide dehydrogenase or flavin adenine dinucleotide through the ETC.

Through targeting of host mitochondrial ETC complexes, we show delayed HCMV replication kinetics and reduced viral titers. Together our data suggests that the ETC of host mitochondria could be a novel target against persistent or slow replicating viruses. Focusing on host machinery that is essential for viral replication minimizes the potential for development of viral resistance.

2. Materials and methods

2.1. Cell culture

Human foreskin fibroblast cells (HFFs) were purchased from American Type Culture Collection (ATCC). HFFs were cultured in Dulbecco's Modified Eagles Medium (DMEM) containing 4.5 g/L glucose and L-glutamine (ThermoFisher) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) at 37 °C and 5% CO_2 unless otherwise specified.

2.2. Viral strains, growth and purification

The HCMV Towne strain (VR-977) was obtained from ATCC. The HCMV TR strain was originally a gift from Dr. David Johnson's lab (Oregon Health and Sciences University, Portland, OR). Virus stocks were grown on confluent HFFs at an MOI = 0.05 and cultured until near complete cell lysis. A standard ultracentrifuge protocol was followed for purification. Briefly, supernatants were collected, spun down to clear cell debris, transferred to autoclaved 25×89mm polyallomer tubes (Seton Scientific) and under laid with 20% sorbitol solution in trissodium (TN) buffer and ultracentrifuged for 2 h at 22k rpm. Pellets were resuspended in TN buffer and stored at −80 °C until use.

2.3. Viral infection

HFFs were plated near confluent as above, serum starved in DMEM containing 4.5 g/L glucose and L-glutamine overnight. The following day, cells were infected at an MOI = 3 with clinical strain HCMV TR, or the lab adapted strain Towne as specified, for 90 min. After 90 min, media was removed and replaced with fresh glucose-free DMEM with 4.5 g/L glucose (high glucose conditions) or 1 g/L glucose (low glucose conditions) containing 10% FBS. Galactose experiments were conducted as described above with 4.5 g/L of galactose replacing glucose. Metformin (3 mM), rotenone (0.1 μm), antimycin (1 μm), oligomycin (1 μm), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (5 μm), succinic acid (10 mM) or ganciclovir (10μM) was added 90 min post HCMV infection to media at indicated concentrations. All chemicals were purchased from Sigma-Aldrich, unless otherwise noted. For pre- and post-treatment experiments, metformin (3 mM) was added to cells 24 h prior to infection, cells were washed and infected for 90 min prior to replacing with fresh media + metformin as described above. The FBS added at 10% contributed ~ 1–1.4×10^{-5} g/L glucose to indicated amount of glucose or galactose.

2.4. Viral titers

Samples were serially diluted and used to infect HFFs in triplicate for determining concentration of viral stocks and in duplicate for inhibitor experiments, all in 96 well plates. After 48 h, cells were washed with PBS, fixed in ice-cold methanol for 5 min, stained with primary mouse anti-IE1 antibody (kind gift of Bill Britt) followed by anti-mouse Alexa

fluor-555-conjugated secondary antibody (ThermoFisher). Plates were imaged at 10X on a Nikon Eclipse TE300 by scanning each well in which 50–150 infected cells could be counted for infectious unit/mL (IU/mL).

2.5. Viability assays

The fixable viability dye eFluor 780 (eBioscience) was used to access apoptosis and toxicity levels during drug treatments. Briefly, cells were plated on 60 mm dishes and treated as described above. At the completion of the experiment, supernatant and trypsinized cells were collected together, centrifuged and resuspended in media containing eFluor 780 following manufacturer's instructions. After 30 min incubation, samples were immediately read on an LSR II.

An MTT assay was also used as follows: HFFs were seeded into 96 well plates in replicates for each treatment, serum starved overnight the following day, and then treated in DMEM containing 10% FBS with selected concentration of drug, vehicle, or positive control (2.5% (v/v) DMSO). After 48 h, media was removed and replaced with serum free, phenol red free MEM brought to 1X, containing 1.09 mM MTT reagent (ThermoFisher), and incubated at 37 °C. After 4 h, MTT containing media was removed and replaced with isopropanol containing 4 mM HCl and 0.1% IGEPAL CA-630, incubated for 15 min at 37 °C, mixed, and absorbance was read at 590 nm on a Biotek μQuant spectrophotometer.

2.6. Immunoblotting

Cells treated as indicated were washed with ice-cold PBS and lysed using RIPA buffer with protease and phosphatase inhibitor cocktails (Sigma). Protein concentrations were quantified using a BCA assay (Pierce). Proteins were separated on 4–12% or 10% polyacrylamide gels (Invitrogen) and transferred onto nitrocellulose membranes using an iBlot device (Invitrogen). Membranes were incubated in TBS +0.1% Tween (TBST) and blocked using 5% BSA (wt/vol) or 5% dried nonfat milk (wt/vol) in TBST to inhibit non-specific antibody binding. Membranes were incubated overnight in 1% blocking solution in TBST and primary antibodies. Membranes were washed in TBST and incubated with secondary antibodies at room temperature for 1 h. Immunoblots were visualized by ECL using a GE Amersham imaging system. Antibodies used were mouse monoclonal anti-HCMV immediate-early (IE), pp65, pp28 (All at 1:200) (Kind gift from Dr. Bill Britt), pp52 (1:250) (Santa Cruz Biotechnology) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:500) (Sigma-Aldrich).

2.7. Respiration measurements

Mitochondrial respiration activity was determined using the Seahorse Flux Analyzer XF^{e}24 (Agilent Technologies, Santa Clara, CA) following the manufacturer's instructions. Briefly, HFFs were seeded on Seahorse 24-well plates and incubated overnight in complete DMEM. Cells were mock- or HCMV-infected with HCMV TR strain (MOI = 3) in fresh media for 48 h. The OCR rates were an average of at least three independent experiments and normalized by cell number. Samples were mixed (3 min), incubated (2 min), and measured (3 min). Mitostress reagents, oligomycin (1 μM), FCCP (1 μM), and rotenone/antimycin (0.5 μM) were injected at indicated time points.

2.8. Statistical analysis

For pharmacological inhibition studies, statistical significance was evaluated using Student's *t*-test with Bonferroni correction. MTT and Seahorse statistical significance was compared to corresponding vehicle sample and evaluated using one-way Anova with Bonferroni correction. Bars represent mean ± SEM and a *P* value < 0.05 was used for statistical significance.

3. Results

3.1. ETC inhibitors significantly reduce HCMV titers

The ETC inhibitors rotenone (complex I), antimycin (complex III) and oligomycin (ATP synthase) were added to HCMV TR infected HFFs to determine the antiviral potential of each drug. Treatments were added to tissue culture medium 1.5 h post infection (hpi). Rotenone, an inhibitor of complex I, and previously shown to inhibit CMV replication (Reeves et al., 2007), reduced HCMV titers by nearly 1 log by 3 days post infection (dpi) (Fig. 1A). Significant inhibition of viral titers continued, through day 6 dpi, however, we observed significant cell death (~60%) at 4 dpi (Fig. 1B). This latter result was expected due to rotenone being an irreversible inhibitor. Antimycin, a complex III inhibitor, showed a nearly 3-fold reduction in HCMV titers at 4 dpi (Fig. 1A). Titers remained significantly lower through day 6, although beginning at 5 dpi, titers began to rebound and slowly approached titers observed under untreated conditions. Cell viability remained high with antimycin treatment (>70% viable) (Fig. 1B). Interestingly, at late time points (120 hpi), antimycin treated cells had greater viability than vehicle treated HCMV infected cells. Oligomycin, an ATPase inhibitor, also showed reduced viral titers at 3 dpi (Fig. 1A). Oligomycin was able to decrease viral titers by ~1 log for the duration of the 6-day measurements. Oligomycin also showed increased cell death at late time points but was not significantly different from vehicle treated HCMV infected cells (Fig. 1B). Similar to antimycin, viral titers rebounded during later time points post oligomycin treatment. These results suggest that targeting components of the ETC can reduce HCMV titers, although multiple treatments might be required to sustain inhibition of viral replication.

3.2. ETC inhibitors delay HCMV early and late protein expression

To further validate the viral inhibition results observed in Fig. 1, time-dependent expression of viral proteins was measured. Lysates from cells infected with HCMV TR were probed against HCMV specific immediate-early (IE), early (pp52), late (pp65) and true late (pp28) viral proteins. No changes in IE expression between mock-treated and cells treated with rotenone, antimycin or oligomycin were observed (Fig. 2A). Interestingly, reduced expression of pp52 in cells treated with ETC inhibitors versus mock-treated was observed (Fig. 2A). Initial expression of pp52 was consistently observed beginning at 1–2 dpi, but total pp52 protein levels were decreased with ETC inhibitor treatment. Total pp52 protein levels of mock- and ETC inhibitor-treated conditions became equal at 4 dpi suggesting that ETC inhibitors delay but do not stop viral protein expression. Expression of pp65 and pp28 were delayed by up to 24 h when treated with ETC inhibitors (Fig. 2A). Specifically, antimycin treatment during HCMV infection resulted in no expression of pp65 until 4 dpi and no expression of pp28 until 5 dpi. This suggests that ETC inhibitors induce bioenergetic

stress and/or prevent infected cells from meeting biomass (protein, lipid, etc.) demands during HCMV replication. Due to reduced or delayed availability of required resources, initiation of defined HCMV replication phases was delayed. These results corroborate our observations in Fig. 1 where we reported reduced viral titers early during replication and a recovery of viral titers at later time points.

3.3. Decreased mitochondrial function correlates with reductions in viral titers

The Seahorse bioanalyzer Mitostress assay (Fig. 3A) was employed to confirm that the ETC compound treatments at the doses given, were reducing mitochondrial function (Fig. 3B and E). Although the chosen inhibitors have been shown to function as mitochondrial respiration inhibitors under uninfected conditions, Seahorse assays at 2 and 6 dpi were used to ensure HCMV infection did not impact inhibitor activity. At 2 dpi, HCMV infection increases mitochondrial basal oxygen consumption rates (OCR), maximal OCR and spare respiratory capacity as described previously by our lab (Combs et al., 2019) (Fig. 3B and C). The addition of rotenone significantly reduced basal respiration and ATP production when comparing HCMV TR versus HCMV TR + rotenone conditions (Fig. 3C). Of note, the spare respiratory capacity was only minimally reduced when infected and treated. Also, rotenone treatment did not completely prevent mitochondrial function as mitochondrial respiration was still detected (Fig. 3C). When we quantitated mitochondrial function using Seahorse assays at 6 dpi, we observed a continued reduction of mitochondrial function in rotenone treated conditions (Fig. 3D). Cells continued using mitochondrial respiration, but basal and maximal respiration, spare respiratory capacity and ATP production were all significantly reduced compared to mock-treated conditions. Alternatively, at 2 dpi, antimycin treated cells exhibited almost no mitochondrial respiration activity (Fig. 3E). Antimycin significantly reduced all mitochondrial measures. When we analyzed 6 dpi conditions, we observed a complete recovery of mitochondrial function in antimycin treated conditions (Fig. 3G). The basal, maximal and spare respiratory capacity of HCMV + antimycin treated cells was similar to levels observed in HCMV infected only cells. These observations suggest that the recovery in viral titers observed in Fig. 1B, may be due to decreasing drug concentrations/efficacy over time. The data did not show significant differences when comparing respiration of mock-infected rotenone- or antimycin-treated cells and HCMV-infected rotenone- or antimycin-treated cells arguing that antimycin and rotenone act similarly in both mock-infected and HCMV-infected cells. Collectively, the data verified that inhibition of mitochondrial respiration and ETC ATP production is decreased. Thus, the strong reduction in all measures of mitochondrial function correlated with reduction in viral titers.

3.4. Diverse HCMV strains are susceptible to ETC inhibitors

To determine whether our observations are strain-specific, the lab-adapted Towne strain of HCMV, a highly passaged strain that exhibits fibroblast tropism, was substituted for HCMV TR in high glucose conditions. Again, a consistent reduction in viral titers was seen beginning at 2 dpi with a single treatment of rotenone, antimycin or oligomycin (Fig. 4A). All treatments displayed significant reductions at some stage of infection. Antimycin displayed the strongest reduction in viral titers, showing an almost 3-fold reduction at 3 dpi (Fig. 4A). Oligomycin showed a ~2-fold reduction at 3 dpi, but minimal changes at other

time points. Rotenone only minimally reduced viral titers. Most samples treated with ETC inhibitors displayed recovered titers by 5 dpi. We also used the mitochondrial uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) in this assay to determine if interference with the mitochondrial membrane potential (MMP) could disrupt ATP production and reduce viral titers. At 3 dpi there was a significant reduction, but generally, FCCP treatment had minimal effect on HCMV replication under these conditions. The inhibition of replication was significant but notably weaker than that observed when using the clinical TR strain, possibly related to differences in TR and Towne strain replication kinetics.

3.5. Metformin reduces HCMV viral titers

Rotenone, antimycin and oligomycin are pharmacological ETC inhibitors that show antiviral activity in our study (Figs. 1–4) but are too toxic to use in humans. Thus, a more translational approach was taken by using metformin, an FDA approved drug that has been reported to inhibit ETC complex I (reviewed in (Fontaine, 2018)). Preliminary studies by Li et al., showed metformin exhibited strong CMV replication suppression (Huifen et al., 2017). Metformin was tested to determine its efficacy and cytotoxicity during HCMV replication. IC_{50} and CC_{50} concentrations were determined to be 0.8 mM (Fig. 5A) and >3 mM respectively. HCMV-TR infected cells were treated with metformin (3 mM) and a significant decrease in the production of virus compared to vehicle treated (DMSO) infected cells was observed (Fig. 5B). The results were very similar to those observed with rotenone treatment, our initial complex I inhibitor. Ganciclovir (10μM) treatment is compared in Fig. 5B, and shows metformin treated conditions exhibit an ~1 log increase in titers compared to ganciclovir. The IC_{50} of ganciclovir is reported to be 1.6 ± 1.5 μM (Hartline et al., 2018). When metformin was tested under low glucose (1 g/L) media conditions, a concentration that more closely represents a physiologically relevant metabolic environment (79–110 mg/dL) (van Enter and von Hauff, 2018), an almost 2-fold reduction in viral titers was observed (Fig. 5C). This trend in viral titer reduction remained consistent through 6 dpi. Furthermore, metformin treatment during HCMV infection in HFFs showed no significant cell death. A trend can be observed at 3 and 5 dpi in which metformin treatment decreased cell death (Fig. 5D).

3.6. Metformin treatment delays early and late viral protein expression

Cellular lysates from the above titer experiments were subjected to Western blot analysis for expression of viral proteins (HCMV-TR) over five days of infection (Fig. 6A). Expression of IE protein was not affected by metformin administration. There was a slight delay in the expression of early protein expression (pp52) and a marked delay in expression of leaky-late (pp65) and true late expression (pp28) (Fig. 6A). These trends were observed when using the ETC inhibitors described earlier in this manuscript (Fig. 2). This suggests that metformin reduces HCMV titers by inhibiting or disturbing an ETC-related function necessary to initiate early/late HCMV gene expression.

3.7. Metformin disrupts ETC activity during HCMV infection

Validation of metformin-mediated inhibition of mitochondrial function using the Seahorse bioanalyzer was again used. At 2 (Fig. 7A) and 6 (Fig. 7B) dpi a Seahorse Mitostress test

was employed to measure mitochondrial function. At 2 dpi, metformin significantly reduced basal OCR in both mock- and HCMV TR-infected samples (Fig. 7A). Maximal OCR was also reduced to similar levels in mock- and HCMV-infected samples, suggesting that metformin was specifically targeting mitochondrial function. The spare respiratory capacity of the mitochondria was increased with metformin treatment in both mock- and HCMV-infected cells but was not significant. Mitochondrial ATP production was almost completely inhibited with the addition of metformin under all conditions. At 6 dpi, we observe a recovery of mitochondrial function in metformin treated cells (Fig. 7B), consistent with our observations using other ETC inhibitors (Fig. 3G). Basal and maximal OCR levels recovered and were equal or higher than levels measured in uninfected, untreated control cells, but were still significantly lower than infected, untreated conditions. ATP production rates increased but were also significantly lower than infected, untreated conditions. Similar to Fig. 3, the data verified that inhibition of mitochondrial respiration and ETC ATP production is decreased when HFFs are treated with metformin regardless of HCMV infection status. This suggests that a single treatment with metformin effectively disrupts mitochondrial function 48 h after treatment, but a single treatment is ineffective 6 days after addition to tissue culture conditions.

3.8. Inhibition of replication by metformin is not strain specific but is sensitive to metabolic conditions

To validate that the results shown in Figs 5–7 are not restricted to the HCMV TR strain, the antiviral effects of metformin were determined using the Towne strain of HCMV (MOI = 3) under high glucose conditions. Although inhibition between vehicle and metformin treated infected cells remained significant, there was only about half a log difference in titer (Fig. 8A). This suggests that metformin inhibition of HCMV viral replication is not strain-specific.

To determine whether metformin could disrupt viral entry or initiation of viral replication and function as a prophylactic, cells were treated overnight with metformin prior to infection with HCMV TR. After a 1.5 h infection period, metformin was again added to the culture media. The addition of metformin pre-infection and post-infection had no noticeable accumulative effect when compared to titer counts after strictly post-infection treatment (Fig. 8B). It also validated our previous data (Figs. 2 and 6) showing no effect on viral intermediate early gene product expression.

Previous studies demonstrated that substituting galactose for glucose as a carbon source in media, forces cells normally conditioned to using high glucose concentrations to utilize mitochondrial respiration for almost all energetic requirements (Reitzer et al., 1979; Aguer et al., 2011). HCMV replicated normally in galactose (25 mM) media but was severely inhibited when treated with metformin (Fig. 8C). The steady decline of titer counts, and visual observation of the cells (data not included) suggested that metformin administration in galactose media results in increased cell death by 3 dpi. The increased cell death observed under these conditions may explain the inhibition of viral replication observed. This data also suggested that metformin is inhibiting HCMV replication primarily through inhibition of mitochondrial respiration and energy production. To further validate that ETC

complex I is important for HCMV replication, we infected HFFs with HCMV TR in low glucose media (1 g/L) and added rotenone (0.1 μM), metformin (3 mM) and metformin + succinic acid (10 mM). Succinate is an ETC complex II intermediate. If ETC complex I is non-essential for HCMV replication, we should observe a rescue of viral titers with succinic acid supplementation as it would allow functional ETC from complex II onward. Low glucose conditions did not adversely affect HCMV TR replication kinetics (Fig. 8D). Results were very similar to observations in normal media (4.5 g/L glucose) shown in Fig. 5C. We did observe significant reductions in viral titers when ETC inhibitors were added. The low glucose conditions did contribute to further reductions in viral titer when treated with rotenone ($\sim 10^3$ Fig. 8D) compared to normal media (10^5 Fig. 1A). Metformin treated conditions repeated our observations from Fig. 5C and succinic acid supplementation did not rescue viral titers (Fig. 8D). Importantly, we did not observe significant differences in cell death between infected cells + metformin and infected cells + metformin + succinic acid (Fig. 8E). Rotenone treatment of HCMV infected cells induced high cell death regardless of glucose concentrations (Fig. 8E compared to Fig. 1B). Together, this suggests that ETC complex I activity is required for efficient HCMV replication.

4. Discussion

The lack of a promising vaccine against HCMV highlights the need for development of novel antivirals against this virus. Despite current HCMV front line drugs being effective, issues of toxicity and viral mutation rendering the antivirals ineffective, continue to present significant clinical problems (Limaye et al., 2000; Lurain and Chou, 2010). This manuscript describes a new potential target against HCMV that minimizes the possibility of viral resistance through mutation. Metabolism is vital for HCMV replication and as our understanding of HCMV metabolic requirements increase, the potential list of antiviral targets should too. Targeting pathways required by the virus, but belonging to the host, could open a multitude of possibilities for future broad-acting antiviral strategies (Hahn et al., 2020).

The data presented here demonstrate that HCMV requires the host cell mitochondria and specifically the ETC for efficient viral replication and suggest the possibility of employing ETC inhibitors as successful antivirals. Targeting the ETC or mitochondrial translation machinery to prevent CMV function has been observed previously but not as an antiviral (Reeves et al., 2007; Huifen et al., 2017; Kaarbo et al., 2011). Regardless of the specific ETC complex targeted, viral titers are reduced (Figs. 1 and 5). This validates the importance of the host ETC during HCMV replication and is consistent with our previously published data showing cells missing mitochondrial DNA (mtDNA) fail to support HCMV replication (Combs et al., 2019). The mtDNA is responsible for encoding many of the proteins required for ETC complex formation. Importantly, this strategy is effective against both clinical and laboratory strains. The implication is that viral genes required for manipulation of host metabolic pathways are critical and highly conserved amongst strains, which strengthens the rationale for targeting the host metabolic machinery.

Most drugs targeting the ETC are too toxic for *in vivo* use. In contrast metformin, a selective inhibitor of ETC complex I, is FDA approved and has a strong safety record in numerous

clinical applications. Our data suggests that metformin is capable of interfering with HCMV replication and the associated reduction in viral titers is mediated by an inhibition of mitochondrial respiration. In support of our data on HCMV, Cheng et al., reported that metformin inhibited Kaposi's sarcoma-associated herpesvirus (KSHV) virion production and viral gene expression (Cheng et al., 2016). Granato et al., used metformin to reduce ROS levels in KSHV infected cells (Granato et al., 2018). By decreasing ROS levels using metformin, KSHV was unable to initiate a lytic cycle. Metformin has been shown to reduce HIV-1 replication, hepatitis B protein production, and cell growth in hepatitis C infected cells (Guo et al., 2021; Xun et al., 2014; Del Campo et al., 2018). The precise mechanism by which metformin inhibits ETC complex I is not known. In a cancer cell model, it is reported that metformin treatment disrupted carbon flow into the tricarboxylic acid (TCA) cycle, thus interfering with citrate dependent *de novo* lipogenesis (Griss et al., 2015). Recent studies highlight the importance of lipid synthesis during HCMV infection (Xi et al., 2019; Koyuncu et al., 2013). Although the ability to suppress TCA cycle associated production of metabolic intermediates required by HCMV to replicate is not directly proven in this study, it is consistent with our published data (Combs et al., 2019). It is possible that inhibition of the ETC prevents accumulation of NAD and/or FAD. NAD may be required during viral replication to prevent accumulation of ROS, assist in signaling (e.g. Ca2+) or as a metabolic regulator of sirtuin expression. FAD may contribute to lipid synthesis. We are continuing these studies to define the mechanisms of these intermediates during HCMV infection. It is intriguing to further speculate that virions propagated under ETC inhibition conditions would differ in composition and infectivity from virions replicated under "normal" conditions. Metformin treatment could also prevent chronic low-grade inflammation associated with CMV-infection as proposed by Chen et al. (2019).

Metformin has been shown to be less effective as an anticancer agent in cancer cells cultured in high levels of glucose (Varghese et al., 2019). A similar phenomenon is observed in our studies. Infection kinetics of the clinical HCMV strain TR, under high physiological concentrations of glucose (25 mM) show a significant but less robust inhibition of replication when compared to low (5.5 mM) glucose concentration conditions (Fig. 5). The lab adapted Towne strain in high glucose conditions (25 mM) also shows a less robust inhibition with metformin treatment (Fig. 8). Low glucose (5.5 mM) media conditions model the physiological concentration of glucose found *in vivo* and does not appear to adversely affect normal HCMV replication. We also show that metformin treatment of cells prior to infection does not hinder viral entry or early phases of viral gene expression (Fig. 8B). This argues that some event during replication of HCMV requires the specific output of mitochondrial respiration. By using galactose as the carbon source, infected cells must rely upon mitochondrial respiration for energy production during replication. When metformin is added under these conditions, HCMV does not replicate, likely due to the inability of HCMV infected cells to meet ATP demands, resulting in cell death. From an ATP centric view, it appears that HCMV replication is not restricted by ATP availability. Metabolic rewiring during HCMV infection includes using alternative carbon sources for ATP generation (Yu et al., 2011). Our data indicates that temporary inhibition of ETC complexes resulting in decreased ATP levels, does not prevent HCMV replication. It has been suggested that the increase in aerobic glycolysis observed during HCMV infection may

be used to generate ATP. Our study did not explore this source of ATP. It has also been reported that influenza replication only consumes ~1% of the host cells available energy (Mahmoudabadi et al., 2017). A deeper understanding of how much and where ATP is generated during HCMV infection is currently being explored.

Combining our understanding of how HCMV impacts host cell metabolism and the functionality of pharmacological agents such as metformin open up new avenues in the search for novel antiviral targets. Currently, our strategy would likely complement existing therapeutic schedules, potentially reducing the duration a patient is required to be on the antiviral. Metabolic inhibitors combined with current antivirals could maximize antiviral efficacy. Alternatively, manipulation of an individual's diet may be advantageous in combination with antiviral therapy. These ideas remain to be explored and tested. Our system, although only tested on HCMV, potentially can be applied to other slow replicating, persistent viruses.

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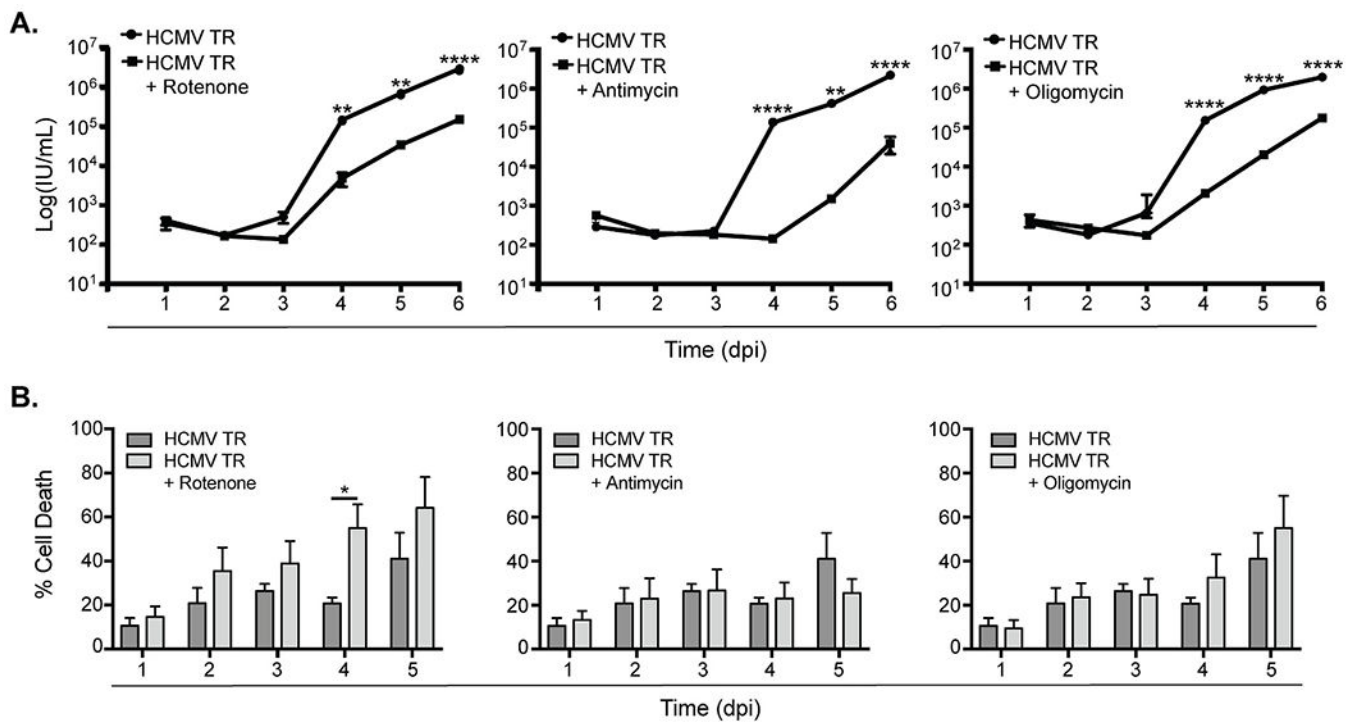
Fig. 1.
ETC inhibitors rotenone, antimycin and oligomycin reduce HCMV titers. (A) HFF cells were infected with HCMV TR for 90 min. Media was changed and ETC inhibitors were added to infected cells. Supernatant was collected every 24 h and quantified for viral presence by titration assays. (B) HCMV-infected and HCMV-infected + ETC inhibitor treated cells were assayed for viability using an eFluor viability marker detected using flow cytometry. N = 3 for all experiments. Bars represent mean ± SEM. *p < 0.05, **p < 0.01, ****p < 0.0001 between HCMV and HCMV + treatment groups. IU/mL = infectious units per milliliter, dpi = days post infection.

A.
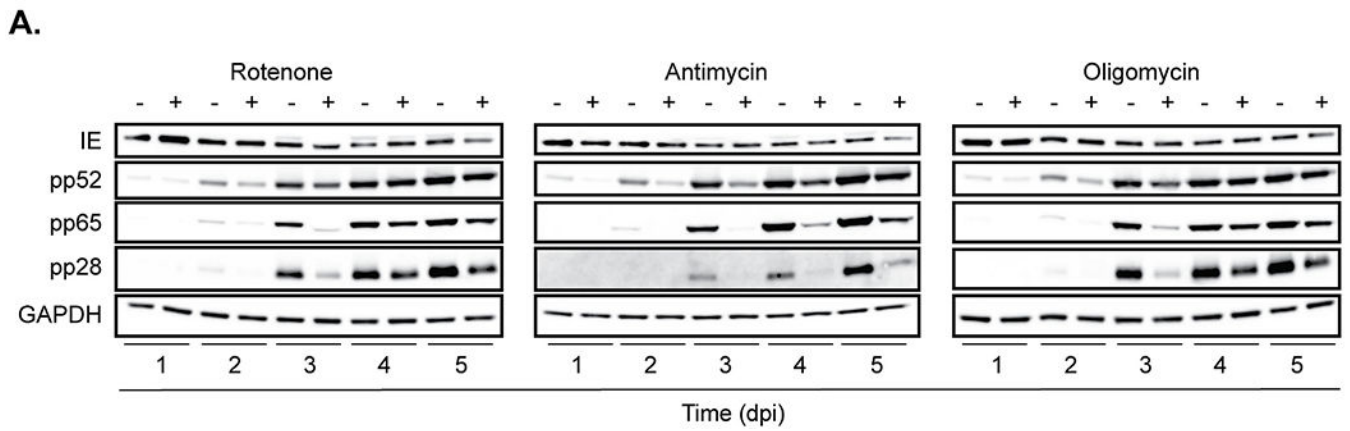
Fig. 2.
ETC inhibitors delay HCMV early and late protein expression. (A) HFFs were infected for 90 min with HCMV TR (MOI = 3) prior to changing to fresh media that includes ETC inhibitors. Protein lysates were collected and probed for HCMV proteins. IE = immediate-early protein, GAPDH = glyceraldehyde 3-phosphate dehydrogenase, dpi = days post infection. Representative of three independent experiments.
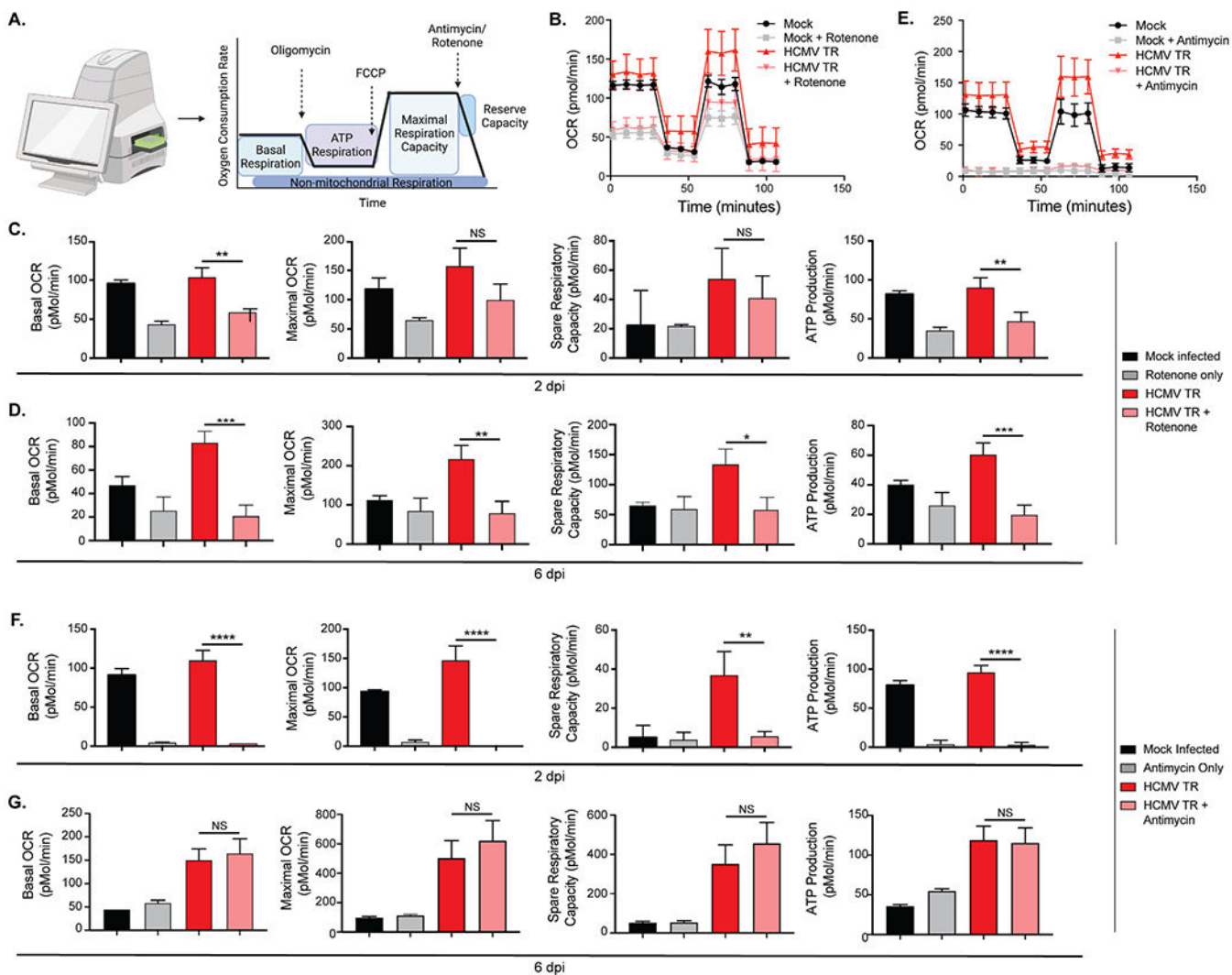
Fig. 3.
ETC inhibitors rotenone and antimycin reduce mitochondrial respiration during HCMV infection. (A) Schematic illustrating the Seahorse Bioanalyzer Mitostress test. (B) Mock- and HCMV-infected cells were mock-treated, or rotenone treated and subjected to a Mitostress test. Samples were analyzed at (C) 2 dpi or (D) 6 dpi. (E) Mock- and HCMV- infected cells were mock-treated, or antimycin treated and subjected to a Mitostress test at (F) 2 dpi or (G) 6 dpi. Figure B and E are representative of 3 experiments. Figure C–D and F-G (N = 3). Bars represent mean ± SEM. *p < 0.05, **p < 0.01, **p < 0.001, ****p < 0.0001. OCR = oxygen consumption rate.
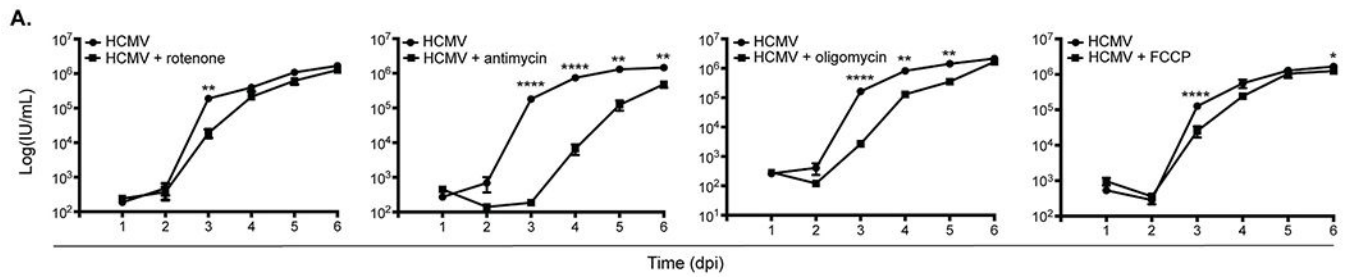
Fig. 4.
ETC inhibitors reduce viral titers in a laboratory strain of HCMV. (A) HCMV Towne infected HFFs were treated with ETC inhibitors. Supernatant was collected. and viral titers were determined. N = 3. Error bars represent mean ± SEM. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. FCCP = carbonyl cyanidetrifluoromethoxyphenylhydrazone.
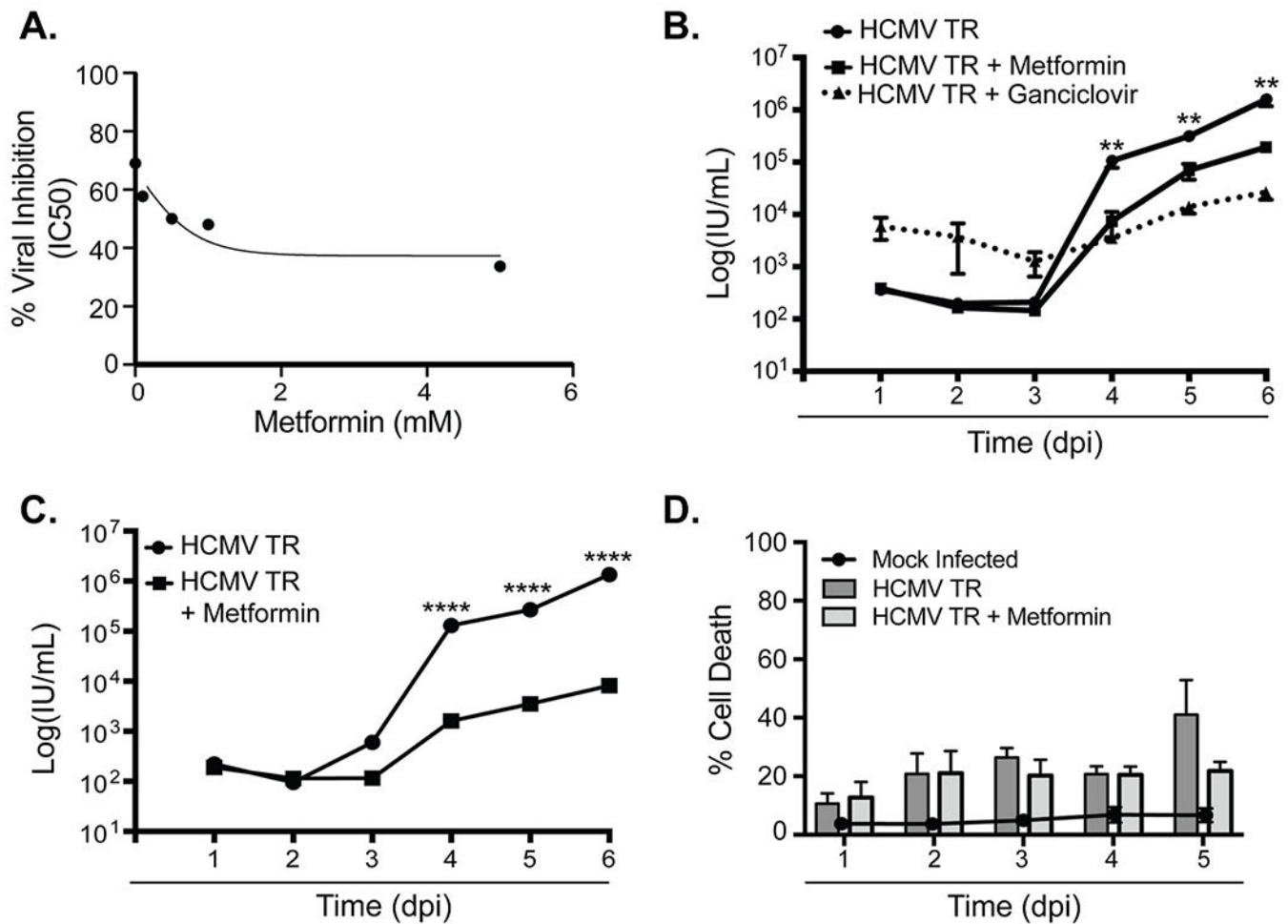
Fig. 5.

Metformin reduction of HCMV TR viral replication is dependent on metabolic environment.
(A) Dose curves were used to determine the IC50 of Metformin during HCMV infection.
(B) Viral titers were determined from HFF cells infected with HCMV TR (MOI = 3)
+/− metformin treatment and ganciclovir control. (C) Metformin treatment of HCMV TR
infected (MOI = 3) HFFs under low glucose (1 g/L) conditions shows stronger inhibition
of viral titers. (D) Viability assay of HCMV infected, metformin treated HFFs using eFluor
viability dye and measured by flow cytometry. N = 3. Bars represent mean ± SEM. **p <
0.01, ****p < 0.0001. IC50 = inhibitory concentration.
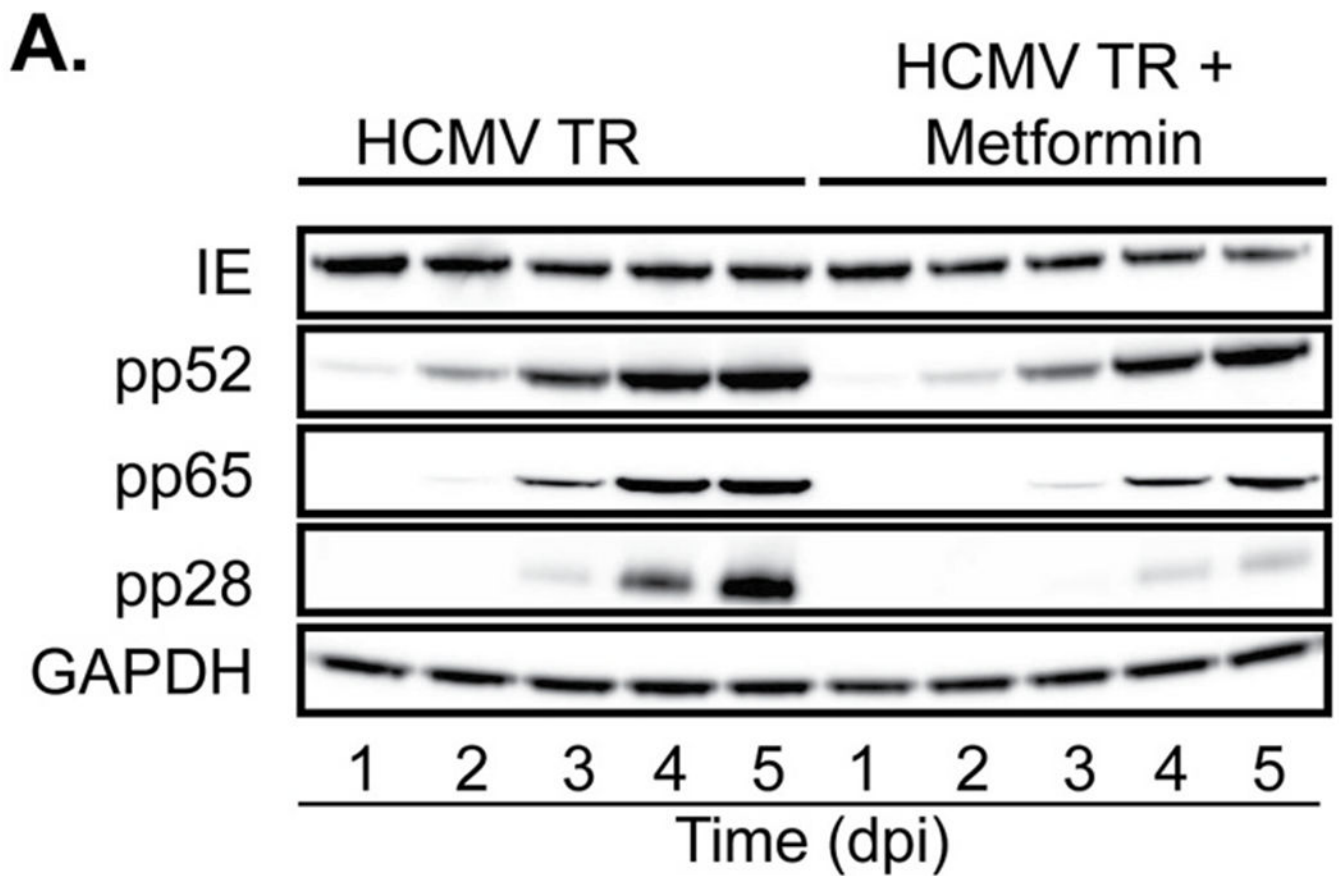
A.

Fig. 6.
Metformin treatment during HCMV TR (MOI = 3) infection of HFFs delays expression of viral proteins. (A) Protein lysates were collected daily after HCMV <u>TR</u> infection and. probed for immediate-early (IE), early, late, and true-late viral proteins. Representative of 3 independent experiments. GAPDH = glyceraldehyde 3-phosphate dehydrogenase, dpi = days post infection.
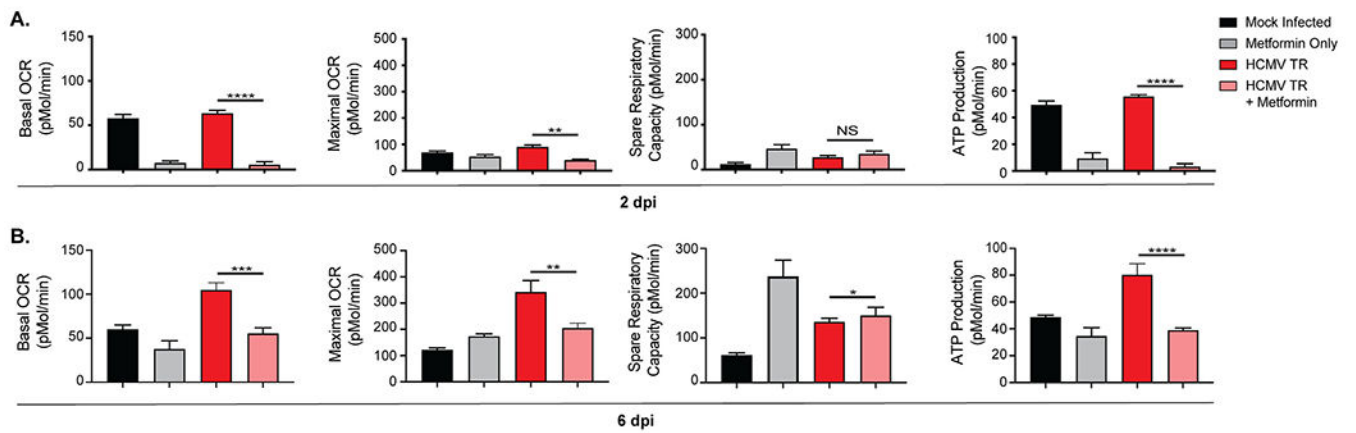
Fig. 7.
Metformin reduces mitochondrial respiration during HCMV infection. Mock- and HCMV TR-infected cells (MOI = 3) +/−metformin. HFFs were assayed for mitochondrial function using a Seahorse XFe24 Bioanalyzer at (A) 2 and (B) 6 dpi. N = 3. Bars represent mean ± SEM. *$p < 0.05$, **$p < 0.01$, **$p < 0.001$, ****$p < 0.0001$. OCR = oxygen consumption rate.
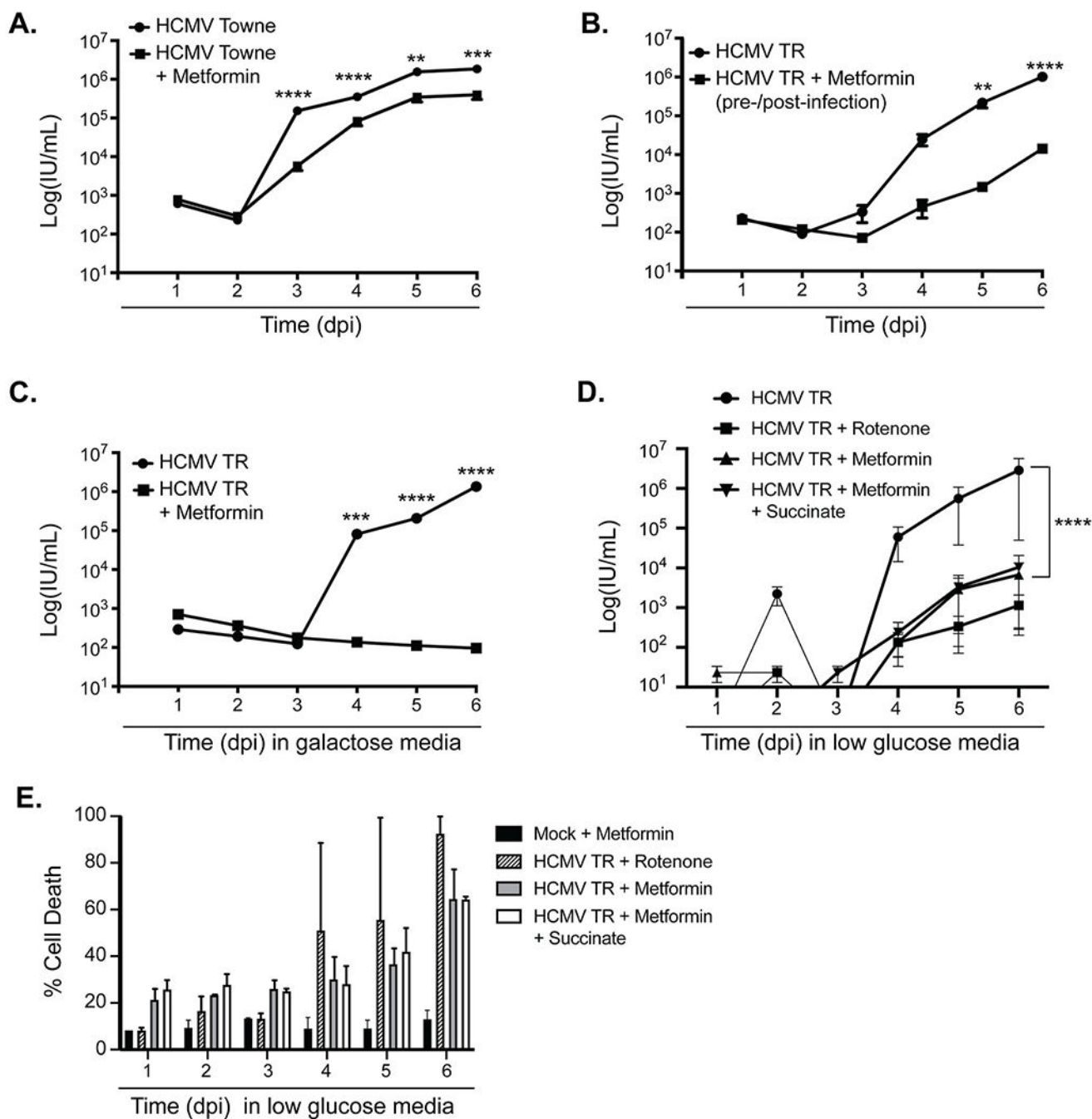
Fig. 8.
Metformin's HCMV antiviral effects are enhanced under nutrient stress. (A) Metformin treatment reduces viral titers when HFFs are infected with the laboratory strain Towne (MOI = 3). (B) Pre- and post-treatment of HCMV TR (MOI = 3) infected HFFs with metformin. (C) Metformin treatment of HCMV TR (MOI = 3) infected HFFs in media containing galactose. (D) HFFs were infected with HCMV TR (MOI = 3) under low glucose conditions (1 g/L). The impact of ETC complex I inhibitors (rotenone and metformin) and ETC complex II intermediate (succinic acid) on viral replication was tested using standard

titration assays. Figures A–D, N = 3, Figure E, N = 2. Bars represent mean ± SEM. **p < 0.01, ***p < 0.001, ****p < 0.0001. dpi = days post infection.