Inhibitors of Anti-apoptotic Bcl-2 Family Proteins Exhibit Potent and Broad-Spectrum Anti-mammarenavirus Activity via Cell Cycle Arrest at G0/G1 Phase

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ABSTRACT
Targeting host factors is a promising strategy to develop broad-spectrum antiviral drugs. Drugs targeting anti-apoptotic Bcl-2 family proteins that were originally developed as tumor suppressors have been reported to inhibit multiplication of different types of viruses. However, the mechanisms whereby Bcl-2 inhibitors exert their antiviral activity remain poorly understood. In this study, we have investigated the mechanisms by which obatoclax (OLX) and ABT-737 Bcl-2 inhibitors exhibited a potent antiviral activity against the mammarenavirus lymphocytic choriomeningitis virus (LCMV). OLX and ABT-737 potent anti-LCMV activity was not associated with their proapoptotic properties but rather with their ability to induce cell arrest at the G0/G1 phase. OLX- and ABT-737–mediated inhibition of Bcl-2 correlated with reduced expression levels of thymidine kinase 1 (TK1), cyclin A2 (CCNA2), and cyclin B1 (CCNB1) cell cycle regulators. In addition, small interfering RNA (siRNA)–mediated knockdown of TK1, CCNA2, and CCNB1 resulted in reduced levels of LCMV multiplication. The antiviral activity exerted by Bcl-2 inhibitors correlated with reduced levels of viral RNA synthesis at early times of infection. Importantly, ABT-737 exhibited moderate efficacy in a mouse model of LCMV infection, and Bcl-2 inhibitors displayed broad-spectrum antiviral activities against different mammarenaviruses and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Our results suggest that Bcl-2 inhibitors, actively being explored as anticancer therapeutics, might be repositioned as broad-spectrum antivirals.

IMPORTANCE
Antiapoptotic Bcl-2 inhibitors have been shown to exert potent antiviral activities against various types of viruses via mechanisms that are currently poorly understood. This study has revealed that Bcl-2 inhibitors’ mediation of cell cycle arrest at the G0/G1 phase, rather than their proapoptotic activity, plays a critical role in blocking mammarenavirus multiplication in cultured cells. In addition, we show that Bcl-2 inhibitor ABT-737 exhibited moderate antimammarenavirus activity in vivo and that Bcl-2 inhibitors displayed broad-spectrum antiviral activities against different mammarenaviruses and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Our results suggest that Bcl-2 inhibitors, actively being explored as anticancer therapeutics, might be repositioned as broad-spectrum antivirals.

KEYWORDS
mammarenavirus, Bcl-2, cell cycle arrest, antiviral, thymidine kinase 1, cyclin A2, cyclin B1, SARS-CoV-2

The Bcl-2 family proteins are critical regulators of apoptosis and are classified into three groups: anti-apoptotic proteins (Bcl-2, Bcl-XL, Bcl-W, MCL-1, and BFL-1/A1), proapoptotic pore-forming proteins (BAX, BAK, and BOK), and proapoptotic BH3-only proteins (BAD, BID, BIK, BIM, BMF, HRK, NOXA, and PUMA) (1). Upon apoptotic stimulation, proapoptotic pore-forming members generate pores within the mitochondrial outer membrane, thus promoting mitochondrial outer membrane permeabilization.
MOMP results in the release of mitochondrial intermembrane space (MIS) proteins, including cytochrome c and SMAC/DIABLO, into the cytoplasm, leading to the activation of the caspase cascade and apoptotic cell death (2). The anti-apoptotic group of Bcl-2 proteins inhibit both proapoptotic groups, pore-forming and BH3-only members, by direct binding to BH3 domains and blocking the progression of the apoptotic signaling cascade before activation of the effector caspases takes place (1, 3). Due to their role in cell survival, anti-apoptotic Bcl-2 proteins are considered attractive targets of tumor-suppressing agents, and a number of inhibitors of Bcl-2 proteins have been developed as chemotherapeutic candidates for cancer treatment (4). Anti-apoptotic Bcl-2 inhibitors mimic the BH3 domain of the proapoptotic Bcl-2 members and occupy the BH3-binding site in anti-apoptotic Bcl-2 proteins, promoting activities of proapoptotic Bcl-2 proteins and apoptosis progression (5). Besides promoting apoptosis, anti-apoptotic Bcl-2 inhibitors have been reported to exert antiviral activities against various types of viruses (6–12). However, the mechanisms by which Bcl-2 inhibitors exert their antiviral activities are not well understood. Several Bcl-2 inhibitors can arrest cells at the G0/G1 phase of the cell cycle, which disrupts normal proliferation and migration of tumor cells (13–15). Notably, several viruses encode proteins that interact with cell cycle–regulating proteins and alter cell cycle progression in different ways (16), but how these interactions can affect virus multiplication remains to be elucidated.

In a previous drug screening, we identified obatoclax (OLX), an antagonist of anti-apoptotic Bcl-2 proteins, as a potent antimammarenaviral drug (17). To investigate the mechanisms whereby Bcl-2 inhibitors could exert their antimammarenavirus activity, we selected the Bcl-2 inhibitors OLX and ABT-737 due to their potent inhibitory effect on the activity of anti-apoptotic Bcl-2 proteins in vitro (18, 19) and in vivo (20–24). We found that the antiviral activity exerted by OLX and ABT-737 against the prototypic mammarenavirus lymphocytic choriomeningitis virus (LCMV) was independent of apoptosis and correlated with OLX- and ABT-737–induced cell cycle arrest at the G0/G1 phase, which was associated with reduced expression levels of multiple cell cycle regulators, including thymidine kinase 1 (TK1), cyclin A2 (CCNA2), and cyclin B1 (CCNB1). Notably, the altered cellular environment caused by cell cycle arrest at G0/G1 interfered with LCMV RNA synthesis at early times of infection. Importantly, ABT-737 exhibited modest in vivo antiviral efficacy reflected by mice infected with strain clone 13 of LCMV and treated with ABT-737 exhibiting less weight loss and lower viral load compared to vehicle-treated infected mice. Both OLX and ABT-737 also exhibited a potent antiviral effect against the mammarenaviruses Junin (JUNV), the causative agent of Argentine hemorrhagic fever (AHF), and Lassa virus live-attenuated vaccine candidate reassortant ML29, as well as against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), suggesting a broad-spectrum antiviral activity of Bcl-2 inhibitors. Our findings have uncovered a novel mechanism by which Bcl-2 inhibitors can exert their antiviral activity, highlighting modulation of the cell cycle as a potential target for development of broad-spectrum antiviral therapeutics.

RESULTS

Effect of Bcl-2 inhibitors on LCMV multiplication in cultured cells. To investigate whether OLX-mediated antimammarenavirus activity was also observed with other inhibitors of anti-apoptotic Bcl-2 family proteins, we compared the effect of the Bcl-2 inhibitors OLX and ABT-737 on multiplication of a recombinant LCMV expressing the ZsGreen (ZsG) reporter gene (rLCMV/ZsG) in A549 cells. The cells were treated with 3-fold serial dilutions of each Bcl-2 inhibitor starting at 2 h before infection. At 48 h postinfection (hpi), virus multiplication was assessed based on ZsG expression levels that were normalized to vehicle (dimethyl sulfoxide [DMSO])–treated controls (Fig. 1A). We also examined the dose-dependent effect of OLX and ABT-737 on cell viability (Fig. 1B). Both OLX and ABT-737 exhibited a potent dose-dependent inhibitory effect on LCMV multiplication with half-maximal effective concentrations (EC50) of 0.01 and
0.40 μM, respectively, and selectivity index (SI; which is equal to half-maximal cytotoxic concentration [CC₅₀]/EC₅₀ values of 113.60 (OLX) and 49.38 (ABT-737) (Fig. 1C).

OLX and ABT-737 were developed as apoptosis-inducing agents, via their inhibitory activities on Bcl-2 family proteins (18–24), to promote the cell death of cancer cells. We therefore investigated whether apoptosis induction caused by Bcl-2 inhibition contributed to the antiviral activities of OLX and ABT-737. We first confirmed that ABT-737 treatment of A549 cells resulted in an increase of both early (annexin V⁺/7-aminoactinomycin D [7-AAD⁻]) and late (annexin V⁺/7-AAD⁺) apoptotic cells (Fig. 2A). Under our experimental conditions, OLX exhibited broad-spectrum auto-fluorescence in a dose-dependent manner, which prevented us from assessing its effect on cell apoptosis by flow cytometry analysis. We further confirmed the apoptosis-inducing effects of both Bcl-2 inhibitors by detection of caspase activity. OLX and ABT-737 significantly increased the activity of caspase-3, a critical component of the apoptotic pathway (Fig. 2B). However, treatment either with the pan-caspase inhibitor zVAD or with the caspase-3 specific inhibitor zDEVD did not affect the antiviral activity of OLX or ABT-737 (Fig. 2C). To rule out the possibility that LCMV infection could interfere with the apoptotic pathway, we determined levels of caspase activity in LCMV-infected cells treated with OLX or ABT-737, or vehicle (DMSO) control, in the presence and absence of the pan-caspase inhibitor zVAD. LCMV infection did not affect Bcl-2 inhibitor–mediated caspase activation, and caspase inhibition activity of zVAD was not altered by LCMV infection (Fig. 2D).

**Cell cycle arrest at the G₀/G₁ phase correlated with the anti-LCMV activity mediated by OLX and ABT-737 Bcl-2 inhibitors.** The mechanisms by which Bcl-2 targeting compounds exert their physiological effects are not well understood and likely involve different cellular responses including cell cycle progression (13–15, 25), autophagy (26), cell metabolism (27), cell migration (28), and apoptosis (29). Bcl-2 inhibitors have been reported to cause cell cycle arrest at the G₀/G₁ phase (13–15). We therefore tested whether altered regulation of the cell cycle contributed to Bcl-2 inhibitor–mediated antiviral activity. We analyzed cell cycle progression in the presence of Bcl-2 inhibitor ABT-737 and a known cell cycle regulator, the cyclin-dependent kinase (CDK) inhibitor dinaciclib (Fig. 3A). Treatment with ABT-737 resulted in substantial cell arrest at the G₀/G₁ phase. As expected, treatment with dinaciclib increased the G2/M

![FIG 1](image-url) Antiviral activity of apoptosis-inducing Bcl-2 inhibitors. (A) Virus propagation in the presence of OLX or ABT-737. A549 cells were treated with serial dilutions of each compound starting 2 h prior to infection (MOI = 0.01) with rLCMV/ZsG. At 48 hpi, ZsG expression levels were measured and normalized to vehicle control (dimethyl sulfoxide [DMSO])–treated controls. (B) Cell viability was determined using the CellTiter-Glo assay after 48 h of compound treatment. The results were normalized to vehicle (DMSO)–treated controls. (C) EC₅₀, CC₅₀, and selectivity index (SI; CC₅₀/EC₅₀) were calculated for OLX and ABT-737.
Due to technical difficulties in flow cytometry analysis caused by OLX autofluorescence property, we examined the impact of OLX on cell cycle modulation by determining its effects on protein expression levels of cell cycle regulators (Fig. 3B). OLX and ABT-737 exhibited similar effects on the expression pattern of cell cycle regulators, reducing expression levels of thymidine kinase 1 (TK1), cyclin A2 (CCNA2), and cyclin B1 (CCNB1). The G2/M arresting reagent, dinaciclib, resulted in a significant increase of TK1 and moderated reduction of CCNA2 and CCNB1. We included treatment with the nucleoside analog ribavirin as a reference for the effects of a broad-spectrum antiviral (30). Ribavirin treatment caused only minor reduction of CCNB1 expression. We next tested whether these cell cycle regulators contributed to LCMV multiplication. Small interfering RNA (siRNA) mediated knockdown of TK1, CCNA2, and CCNB1, as well as Bcl-2, resulting in significant reduction of virus multiplication (Fig. 3C). The knockdown level of each factor was confirmed by Western blot analysis, revealing that Bcl-2 knockdown caused significant decrease in expression levels of TK1, CCNA2, and CCNB1 (Fig. 3D), suggesting that Bcl-2 is an upstream phase population.
FIG 3 Effects of Bcl-2 inhibitor-induced G0/G1 arrest on LCMV multiplication. (A) A549 cells were treated with the indicated compounds and concentrations for the indicated times, followed by addition of BrdU to the cell culture medium for 1 h before harvesting cells. Cell cycle positions and active DNA synthesis in fixed cells were analyzed by the correlated levels of total DNA and incorporated BrdU using flow cytometry. (i) Plots are representative gatings for each compound treatment. (ii) Cells (%) at each phase of the cell cycle after 24 and 48 h of treatment with each compound. (B) Whole-cell lysates were collected at 48 h posttreatment, and protein expression levels were determined by Western blotting. (C, D) 293T cells were transfected with the indicated siRNAs and for 4 h later infected (MOI = 0.01) with rLCMV/ZsG. At 48 hpi, levels of infectious progeny in tissue culture supernatants (C) and protein expression levels (D) were determined by FFA and Western blotting, respectively. N.C, negative control. (E) A549 cells were pretreated with the indicated compounds and infected with rLCMV/ZsG. At 48 hpi, the cell cycle was analyzed using the BrdU assay. Treatment was as follows: OLX (0.1 μM), ABT-737 (2 μM), dinaciclib (5 μM), and ribavirin (100 μM). Statistical significance was calculated by analysis of variance (ANOVA). *, P < 0.05; **, P < 0.002; ***, P < 0.0002; ****, P < 0.00001. LCMV, lymphocytic choriomeningitis virus.
modulator of these cell cycle regulators associated with progression from the S to G2/M phases. Modulation of the cell cycle has been documented as a strategy used by several viruses to enhance their replication (16). To assess whether LCMV infection influenced cell cycle regulation, we infected cells with LCMV in the presence of the indicated compounds and at 48 hpi determined the distribution of cells at each phase of the cell cycle (Fig. 3E). LCMV infection did not affect cell cycle regulation of untreated or ABT-737–treated cells.

Effect of Bcl-2 inhibition on LCMV RNA synthesis. We previously reported that OLX treatment caused a significant reduction on LCMV and LASV RNA levels at 48 hpi (17). This finding, however, reflected the effect of OLX on the steady-state levels of viral RNA over multiple rounds of infection. To gain a better understanding of how Bcl-2 inhibitors might modulate viral RNA synthesis, we examined the effect of OLX and ABT-737 on viral RNA synthesis at early times of infection. We treated cells with the indicated compounds for 24 h before LCMV infection and determined levels of viral genome and antigenomic RNA species at 2 and 4 hpi (Fig. 4). Bcl-2 inhibitors did not affect significantly viral RNA synthesis at 2 hpi. In contrast, at 4 hpi, viral RNA levels were significantly reduced in OLX- and ABT-737–treated cells (Fig. 4A and B). Levels of genomic RNA at 4 hpi increased about 3-fold compared to the levels at 2 hpi (Fig. 4A). Our antigenomic RNA detection system targeted both antigenomic RNA and mRNA species. Levels of antigenomic RNA were 6-fold higher at 4 hpi than at 2 hpi, which reflected newly synthesized nucleoprotein (NP) mRNA (Fig. 4B). Ribavirin slightly increased levels of viral RNA at 2 hpi but at 4 hpi exhibited the expected inhibitory effect on viral RNA synthesis.

Effect of the Bcl-2 inhibitor ABT-737 on LCMV infection in vivo. We selected ABT-737 for in vivo experiments due to its better characterized toxicology and pharmacology. Based on published data, we initially tested two different doses of ABT-737, 20 and 100 mg/kg/day, to determine the appropriate dose for our mouse model of LCMV infection. High-dose treatment (100 mg/kg/day) of ABT-737 caused weight loss of about 15% in mock-infected mice and more severe clinical symptoms in LCMV-infected mice than in vehicle-treated mice (Fig. 5A). We therefore used the lower dose of ABT-737 (20 mg/kg/day) to test its antiviral efficacy in vivo. This low-dose treatment with ABT-737 resulted in minimal weight loss in mock-infected mice but exhibited only moderate antiviral activity in vivo using a mouse model of LCMV infection. We infected adult C57BL/6 mice with a high dose (2 × 10⁶ PFU/mouse) of the immunosuppressive CL-13 variant of the Armstrong strain of LCMV. Infection of adult C57BL/6 mice with a high dose of CL-13 causes transient body weight loss and persistent viremia (31). ABT-737–treated mice showed less body weight loss and faster recovery compared to the vehicle control group (Fig. 5B). Moreover, we observed moderate reduction of viral load in ABT-737–treated mice (Fig. 5C).
Assessing the broad-spectrum antiviral activity of Bcl-2 inhibitors. Host-targeting antiviral drugs are likely to cover broader viral genotypes and potentially exhibit broad-spectrum antiviral activity across viruses from different families. To assess the antiviral spectrum of Bcl-2 inhibitors, we examined the effect of OLX and ABT-737 on the multiplication of two additional mammarenaviruses, the live-attenuated vaccine (LAV) strain, Candid#1, of the New World mammarenavirus Junin (JUNV) (Fig. 6A), and the LASV LAV candidate reassortant ML29, carrying the L segment from the nonpathogenic Mopeia virus and the S segment from LASV (Fig. 6B). For both Candid#1 and ML29, we used their trisegmented versions expressing the green fluorescent protein (GFP) reporter gene (32). In addition, we examined the effect of OLX and ABT-737 on SARS-CoV-2 (Fig. 6C). Both OLX and ABT-737 exhibited a potent dose-dependent antiviral activity against Candid#1, ML29, and SARS-CoV-2.

**DISCUSSION**

Host-targeting antiviral drugs represent an attractive strategy to combat viral infections, as intrahost virus evolution is unlikely to result in viral variants able to escape from inhibitors that disrupt the activity of host cell factors required for the completion of viral replication.
of the virus life cycle. Moreover, related viruses are likely to rely on the same host machinery, thus providing an opportunity for the development of broad-spectrum antiviral therapeutics. Several Bcl-2 targeting small molecules have been identified as potential antiviral drugs against different types of viruses (6–12). However, the mechanisms by which these Bcl-2 inhibitors exerted their antiviral activity are not well understood. In this study, we examined the mechanisms whereby the Bcl-2 inhibitors OLX and ABT-737 exerted their potent anti-LCMV activity. We found that the potent anti-LCMV activity of OLX and ABT-737 was not associated with their proapoptotic properties but rather their ability of inducing cell cycle arrest at the G0/G1 phase. In contrast, the effect of ABT-737 on production of infectious progeny at late times of infection with several flaviviruses was found to be mediated by ABT-737–induced cell death rather than a direct antiviral effect (33).

Small molecules targeting the Bcl-2 family proteins were first developed for cancer treatment, as apoptotic cell death caused by inhibition of anti-apoptotic Bcl-2 proteins contributes to reduced tumor progression (4, 5). We therefore examined whether apoptosis induced by Bcl-2 inhibitors contributed to OLX and ABT-737 anti-LCMV activity. Our finding that OLX and ABT-737 anti-LCMV activity was not associated with their proapoptotic properties but rather their ability of inducing cell cycle arrest at the G0/G1 phase. In contrast, the effect of ABT-737 on production of infectious progeny at late times of infection with several flaviviruses was found to be mediated by ABT-737–induced cell death rather than a direct antiviral effect (33).

![Graph](image-url)

**FIG 6** Broad-spectrum antiviral activity of Bcl-2 inhibitors. (A to C) Normalized virus propagation in the presence of OLX or ABT-737. Vero cells were treated with serial dilutions of each compound starting 2 h prior to infection with r3Can/GFP (A), r3ML29/GFP (B), or SARS-CoV-2 (C). At 72 hpi, GFP expression levels in infected cells (A, B) or titers of infectious SARS-CoV-2 in tissue culture supernatants as determined by IFFA (C) were measured and normalized to vehicle (DMSO)–treated controls.
may also contribute to their tumor-suppressing properties. Accordingly, several anti-cancer compounds that target Bcl-2 have been reported to arrest cell cycle progression, and the corresponding suppression of cell growth has been shown to play an important role in their antitumor activity (13–15, 25). We therefore investigated whether OLX- and ABT-737–mediated effects on the regulation of the cell cycle contributed to their anti-LCMV activity. We observed that Bcl-2 inhibitor treatment arrested cells at the G0/G1 phase, which correlated with a significant reduction in expression levels of cell cycle regulators TK1, CCNA2, and CCNB1 (Fig. 3). Consistent with their distinct roles, these cell cycle regulators have been shown to be expressed at distinct phases of the cell cycle: TK1 expression starts to increase in the early S phase (36). CCNA is elevated at the G2 phase, and CCNB is highly expressed at the M phase (37). Treatment with OLX and ABT-737 resulted in significant reduction of TK1 expression, consistent with the cells being arrested at the G0/G1 phase before entering S phase (Fig. 3B). In contrast, treatment with dinaciclib, known to promote G2/M phase arrest, resulted in highly increased TK1 expression. Our finding that OLX and ABT-737 treatment significantly decreased expression levels of CCNA2 and CCNB1 (Fig. 3B) further supported that expression of G2/M phase regulators was prevented in the presence of G0/G1 promoting Bcl-2 inhibitors. Our finding that siRNA-mediated knockdown of cell cycle regulators TK1, CCNA2, and CCNB1 resulted in reduced levels of virus multiplication suggested that these factors likely play a role in OLX- and ABT-737–mediated anti-LCMV activity (Fig. 3C). We found that siRNA-mediated knockdown of Bcl-2 resulted in the strongest reduction on levels of LCMV multiplication, which was associated also with reduced levels of TK1, CCNA2, and CCNB1 (Fig. 3D), suggesting that Bcl-2 is an upstream regulator of TK1, CCNA, and CCNB expression.

Several viruses regulate the host cell cycle to create a favorable environment for their replication (16). Thus, influenza A virus (IAV) nonstructural protein 1 (NS1) was found to promote a G0/G1 arrest in infected cells via inhibition of the RhoA-pRb signaling cascade to create favorable conditions for viral replication (38, 39). Likewise, SARS-CoV and the murine CoV mouse hepatitis virus (MHV) have been shown to induce G0/G1 arrest in infected cells to favor their multiplication (40, 41). However, LCMV infection did not affect either cell cycle progression or Bcl-2 inhibitor–mediated G0/G1 arrest (Fig. 3E), and in contrast to IAV and CoVs, cell cycle arrest at the G0/G1 phase generates a cellular environment that contributes to limiting LCMV multiplication. Intriguingly, treatment with ribavirin resulted also in increased numbers of cells at the G0/G1 phase (Fig. 3E), suggesting that cell cycle modulation might be another mechanism, in addition to those previously documented (30, 42), by which ribavirin exerts its broad-spectrum antiviral activity.

Several viruses were selected to examine the anti-LMCV activity of Bcl-2 inhibitors in vivo, as ABT-737 safety and efficacy in vivo have been documented for several tumor models (21–24). Under our experimental conditions, high-dose treatment of ABT-737 (100 mg/kg/day) caused significant weight loss (~15%) in mock-infected mice (Fig. 5A). Moreover, infected mice treated with the high dose of ABT-737 rapidly developed severe clinical symptoms resulting in significant mortality (50% death within 4 days postinfection). In contrast, the lower dose of ABT-737 (20 mg/kg/day) resulted in only modest weight loss at early times
after starting treatment of LCMV-infected mice (Fig. 5B). Importantly, at later times postinfection, ABT-737 treatment resulted in faster weight recovery compared to that of the vehicle-treated control mice (Fig. 5B). In addition, ABT-737 treatment resulted in reduced serum viral load throughout the duration of the observation time with the experimental endpoint at 17 days postinfection (Fig. 5C). Toxicity associated with Bcl-2 targeting small molecules can negatively affect their in vivo efficacy as antiviral drugs. Hence, further studies are needed to find modifications that could overcome the toxicity of Bcl-2 targeting drugs. Recent studies suggest that implementation of proteolysis-targeting chimera (PROTAC) with Bcl-2 inhibitors could address this issue. PROTACs are modified small molecules in which target molecules are linked to an E3 ligase ligand, promoting ubiquitination with Bcl-2 inhibitors. The PROTAC form, DT2216, resulted in significantly reduced cytotoxicity but improved antitumor activity (43).

OLX and ABT-737 exhibited antiviral activity also against two other mammarenaviruses, ML29, and the most distantly related New World JUNV. In addition, consistent with recent published findings, we found that OLX exerted a strong inhibitory effect on SARS-CoV-2 multiplication (Fig. 6). These results suggest the possibility that Bcl-2 inhibitors actively being explored as anticancer therapeutics could be repositioned as broad-spectrum antivirals.

MATERIALS AND METHODS

Cells and viruses. Hono sapiens A549 (ATCC CCL-185), 293T (ATCC CRL-3216), and Grivet (Chlorocebus aethiops) Vero E6 (ATCC CRL-1586) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (ThermoFisher Scientific, Waltham, MA) containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 µg/ml of streptomycin, and 100 U/ml of penicillin. The immunosuppressive strain of LCMV, clone 13 (CL-13) (48), recombinant LCMV expressing Zanthurus sp. green fluorescent protein (ZsG) fused to nucleoprotein via a P2A ribosomal skipping sequence (rLCMV/ZsG-P2A-NP, referred to as rLCMV/ZsG) (44); the trisegmented form of the live attenuated vaccine strain Candid#1 of JUNV expressing GFP (rJUNV/GFP) (45); the trisegmented form of reasortant ML29 expressing GFP (r3ML29/GFP) (46) have been described previously. SARS-CoV-2 USA-WA1/2020 (GenBank accession number MN985325.1) was purchased from AstaTech Inc. (Bristol, PA)

Cell cytotoxicity assay and CC50 determination. Cell viability was assessed using the CellTiter 96 AQuueous One Solution Reagent (Promega, Madison, WI). This method determines the number of viable cells based on conversion of formazan product from 3-(4,5-dimethylthazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolim by NADP (NADPH) or NADP (NADH) generated in living cells. A549 cells were plated on 96-well clear-bottomed black plates (2.0 × 104 cells/well). Serial dilutions (3-fold) of each compound were added to cells, and at 48 h after drug treatment, CellTiter 96 AQuueous One Solution Reagent was added and incubated for 15 min. Absorbance was measured at 490 nm using an enzyme-linked immunosorbent assay (ELISA) reader (SPECTRA max plus 384; Molecular Devices, Sunnyvale, CA). The resulting optical densities were normalized with dimethyl sulfoxide (DMSO) vehicle control group, which was adjusted to 100%. Half-maximal cytotoxic concentrations (CC50) were determined using Prism (GraphPad, San Diego, CA).

Determination of EC50 and SI. The cells were plated on 96-well clear-bottomed black plates (2.0 × 104 cells/well) and incubated for 20 h at 37°C and 5% CO2. The cells were pretreated 2 h before infection with 3-fold serial dilutions of each compound. The cells were infected (multiplicity of infection [MOI] = 0.01) with rLCMV/ZsG-P2A-NP in the presence of compounds. At 48 h postinfection (hpi), the cells were fixed with 4% paraformaldehyde, the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI), and ZsG expression was determined by flow cytometry using a flow cytometer (Cytex Aurora, Cytex, Fremont, CA), and the data were analyzed using FlowJo (FlowJo LLC, Ashland, OR). Caspase activity was determined using the Caspase-Glo 3/7 assay system (Promega, Madison, WI) according to the manufacturer’s instructions. The values were normalized with total cellular protein determined with the Pierce BCA protein assay kit (ThermoFisher Scientific).
Cell cycle studies. To analyze stages of the cell cycle, a bromodeoxyuridine (BrdU) assay kit (APC BrdU flow kit; BD Pharmingen) was used as directed by the manufacturer’s instructions. Briefly, the cells were labeled by adding BrdU (final concentration, 10 μM) and incubated for 1 h at 37°C and 5% CO2. After harvesting, the cells were fixed and permeabilized followed by treatment of DNase to expose incorporated BrdU. Next, the cells were stained with allophycocyanin (APC)–conjugated anti-BrdU antibody and 7-AAD. The samples were analyzed by flow cytometry (Cytek Aurora) and FlowJo (FlowJo LLC).

Western blotting. Whole-cell lysates were prepared in radioimmunoprecipitation assay (RIPA) lysis buffer ( thermoFisher Scientific). After sonication for 30 s, the samples were denatured for 10 min at 95°C. 30 μg of each sample was separated by SDS-PAGE, transferred to the polyvinylidene difluoride (PVDF) membrane ( Immobilon PVDF membrane; Millipore Sigma, Burlington, MA), and immunoblotted with BCL-2, TK1, CCNA2, CCNB1, and β-actin antibodies (Cell Signaling Technology, Danvers, MA). The bands were visualized with the chemiluminescent substrate ( thermoFisher Scientific).

Gene knockdown by siRNA. 293T cells were transfected with 10 nM each siRNA using the Lipofectamine RNAiMAX transfection reagent ( thermoFisher Scientific), according to the manufacturer’s instructions. The following predesigned ON-TARGET plus siRNA oligonucleotides were purchased from Horizon Discovery (Cambridge, UK): BCL2 (siRNA ID: J-003307-16); TK1 (siRNA ID: J-006787-09); CCNA2 (siRNA ID: J-003205-10); and CCNB1 (siRNA ID: J-003206-09).

Virus titration. Virus titers were determined by focus-forming assay (FFA) (47). Serial dilutions of samples (10-fold) were used to infect Vero E6 cell monolayers in 96-well plates (2 × 104 cells/well). At 20 hpi, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline. The foci of cells infected with rLCMV/ZsG were determined by epifluorescence of fluorescent reporter gene expression. The foci of cells infected with wild-type LCMV were identified by rat monoclonal antibody VL4 against NP (Bio X Cell, West Lebanon, NH) conjugated to Alexa Fluor 488. SARS-CoV-2 titers were determined by using an immune focus-forming assay (IFFA). Vero E6 cells (3 × 104 cells/well, 96-well plate format, in triplicate) were infected with serial viral dilutions (100–μl final volume/well). At 20 hpi, the cells were fixed overnight with 10% paraformaldehyde in phosphate-buffered saline. For immunostaining, the cells were permeabilized with 0.5% (vol/vol) Triton X-100 in phosphate-buffered saline (PBS) for 15 min at room temperature and immunostained using the SARS-CoV–cross-reactive N protein 1C7/C7 monoclonal antibody (1 μg/ml), followed by reaction with a goat anti-mouse conjugated to Alexa Fluor 488.

RT-qPCR. The cells were infected with rLCMV/Zsg-PA2-NP in the presence of indicated compounds or vehicle control DMSO. Total cellular RNA was isolated using TRI reagent (TR 118) (MRC, Cincinnati, OH) according to the manufacturer’s instructions. Total RNA (1 μg) was reverse-transcribed to cDNA using the SuperScript™ IV first-strand synthesis system ( thermoFisher Scientific). To make strand-specific cDNAs, target primers specific to an genomic or antigenomic strand were designed. Target sequences were amplified and quantified by quantitative PCR (qPCR) with the primer sets listed in the following section.

Primers. The following primers were used: 5′-CAGGGTGCAAGTGGTGTGGTAAGAG-3′ (genomic strand) and 5′-CGAGAACTGCTTTCAAGGGCTC3′ (antigenomic strand) for the reverse-transcription (RT) reaction and 5′-CAGAAATGTGGATGCTGGACTGC-3′ (forward) and 5′-CGACCTTGGGTGTCCCTACAG-3′ (reverse) for qPCR LCMV NP.

Animal studies. Adult (8 weeks old) C57BL/6 inbred laboratory mice (Scripps Research breeding colony) were inoculated intravenously (IV) with LCMV Ct-13 (2 × 105 focus-forming units [FFU]) and treated with ABT-737 (20 mg/kg/day) or vehicle control (30% propylene glycol, 5% Tween 80, and 5% dextrose in water), administered intraperitoneally. Treatment was administered daily from days 1 to 17 postinfection. The mice were monitored daily for development of clinical signs, weight loss, and survival. Sera were collected at day 3, 6, and 17. All animal experiments were done under protocol 09-0137-4 approved by the Scripps Research Institute Institutional Animal Care and Use Committee (IACUC).

ACKNOWLEDGMENTS

This work was supported by grants AI125626 and AI128556 from the NIAID, NIH. This is Manuscript 30121 from the Scripps Research Institute.

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