AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology

The Antiviral Alkaloid Berberine Reduces Chikungunya Virus-Induced Mitogen-Activated Protein Kinase Signaling

Finny S. Varghese,[a] Bastian Thaa,[b] Siti Naqiah Amrun,[c] Diane Simarmata,[c] Kai Rausalu,[d] Tuula A. Nyman,[e] Andres Merits,[d] Gerald M. McInerney,[b] Lisa F. P. Ng,[c] Tero Ahola[a]

Department of Food and Environmental Sciences, University of Helsinki, Helsinki, Finland[a]; Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden[b]; Singapore Immunology Network (SIgN), Agency for Science, Technology and Research (A*STAR), Singapore[c]; Institute of Technology, University of Tartu, Tartu, Estonia[d]; Institute of Biotechnology, University of Helsinki, Helsinki, Finland[e]

ABSTRACT

Chikungunya virus (CHIKV) has infected millions of people in the tropical and subtropical regions since its reemergence in the last decade. We recently identified the nontoxic plant alkaloid berberine as an antiviral substance against CHIKV in a high-throughput screen. Here, we show that berberine is effective in multiple cell types against a variety of CHIKV strains, also at a high multiplicity of infection, consolidating the potential of berberine as an antiviral drug. We excluded any effect of this compound on virus entry or on the activity of the viral replicase. A human phosphokinase array revealed that CHIKV infection specifically activated the major mitogen-activated protein kinase (MAPK) signaling pathways extracellular signal-related kinase (ERK), p38 and c-Jun NH_2-terminal kinase (JNK). Upon treatment with berberine, this virus-induced MAPK activation was markedly reduced. Subsequent analyses with specific inhibitors of these kinases indicated that the ERK and JNK signaling cascades are important for the generation of progeny virions. In contrast to specific MAPK inhibitors, berberine lowered virus-induced activation of all major MAPK pathways and resulted in a stronger reduction in viral titers. Further, we assessed the *in vivo* efficacy of berberine in a mouse model and measured a significant reduction of CHIKV-induced inflammatory disease. In summary, we demonstrate the efficacy of berberine as a drug against CHIKV and highlight the importance of the MAPK signaling pathways in the alphavirus infectious cycle.

IMPORTANCE

Chikungunya virus (CHIKV) is a mosquito-borne virus that causes severe and persistent muscle and joint pain and has recently spread to the Americas. No licensed drug exists to counter this virus. In this study, we report that the alkaloid berberine is antiviral against different CHIKV strains and in multiple human cell lines. We demonstrate that berberine collectively reduced the virus-induced activation of cellular mitogen-activated protein kinase signaling. The relevance of these signaling cascades in the viral life cycle was emphasized by specific inhibitors of these kinase pathways, which decreased the production of progeny virions. Berberine significantly reduced CHIKV-induced inflammatory disease in a mouse model, demonstrating efficacy of the drug *in vivo*. Overall, this work makes a strong case for pursuing berberine as a potential anti-CHIKV therapeutic compound and for exploring the MAPK signaling pathways as antiviral targets against alphavirus infections.

Chikungunya virus (CHIKV) is the causative agent of chikungunya fever, a disease spread by *Aedes* mosquitoes and characterized by a sudden onset of febrile illness, nausea, headache, and most importantly, severe and persistent musculoskeletal pain (1). It reemerged in tropical Asia and Africa 1 decade ago and since 2013 has infected more than 1.5 million people in the Americas (2). So far, no licensed vaccine or antiviral treatment exists to counter this disease. Over the years, many research groups have focused on antiviral drug discovery against CHIKV, but very few of these potential antiviral candidates have been characterized in animal models to demonstrate their *in vivo* efficacy (3, 4).

CHIKV is grouped in the *Alphavirus* genus (family *Togaviridae*), along with viruses such as *O'nyong'nyong virus* (ONNV) and *Sindbis virus* (SINV), which cause similar disease in humans, and low-pathogenic viruses such as *Semliki Forest virus* (SFV). The CHIKV genome is a single-stranded positive-sense RNA, ~12 kb in length, comprising two open reading frames that encode the nonstructural and structural proteins of the virus, respectively. Upon infection, nonstructural proteins 1 to 4 (nsP1 to nsP4) are translated as a polyprotein from the genomic RNA, following which they induce membrane invaginations called spherules that act as active viral replication complexes. After the initial minus strand synthesis, the replication complex switches over to synthesis of full-length genomic RNA as well as the subgenomic RNA, from which the structural proteins (capsid and envelope proteins) are translated. The viral genomic RNA is then packaged into capsids and transported to the plasma membrane, the site of virus budding (5, 6).

Virus infection typically manipulates cellular metabolism and signaling pathways to favor virus replication. CHIKV has been

Received 12 July 2016 **Accepted** 10 August 2016

Accepted manuscript posted online 17 August 2016

Citation Varghese FS, Thaa B, Amrun SN, Simarmata D, Rausalu K, Nyman TA, Merits A, McInerney GM, Ng LFP, Ahola T. 2016. The antiviral alkaloid berberine reduces chikungunya virus-induced mitogen-activated protein kinase signaling. J Virol 90:9743–9757. doi:10.1128/JVI.01382-16.

Editor: M. S. Diamond, Washington University School of Medicine

Address correspondence to Finny S. Varghese, finny.varghese@helsinki.fi, or Tero Ahola, tero.ahola@helsinki.fi.

shown to modulate the prosurvival phosphatidylinositol-3 kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) axis (7), endoplasmic reticulum (ER) stress response pathways (8), and pattern recognition receptor-mediated innate immune pathways (9, 10). However, one major type of signaling network underexplored in the context of alphavirus infection is the mitogen-activated protein kinase (MAPK) signaling. The MAPKs are a group of signal-transducing proteins expressed ubiquitously in most mammalian cell types, primarily mediating the host cell response to diverse external stimuli. The basic structure of the typical MAPK signaling pathway follows a three-tiered cascade of activating kinases, generically termed MAPKKK (MAPK kinase kinase), MAPKK (MAPK kinase), and MAPK, to phosphorylate and activate a plethora of cytoplasmic and nuclear substrates, usually transcription factors with roles in cell growth, differentiation, proliferation, migration, and apoptosis (reviewed in references 11 and 12). The major MAPKs are extracellular signal-related kinase (ERK), p38, and c-Jun NH_2-terminal kinase (JNK) (see schematic in Fig. 6A).

The ERK pathway is activated primarily by growth factors at the cell surface. This signal is relayed down through Ras and Raf (the initiating MAPKKK in this cascade) to MEK (MAP/ERK kinase), which in turn phosphorylates ERK1/2. Upon stimulation, ERK phosphorylates multiple substrates like c-Fos and Elk1, which are involved in the regulation of cell cycle progression and play a prosurvival role (13). The p38 MAPK and JNK pathways are predominantly activated by various environmental stress signals like UV irradiation as well as inflammatory cytokines. p38 MAPK has been reported to be mostly an activator of the immune response to proinflammatory stimuli like tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) as well as a key role player in the induction of apoptosis by cellular stress (12, 14). JNK is a signal transducer to maintain a prosurvival state or the expression of a proinflammatory response as well as apoptotic signals (12). Despite its importance in the replication of many viruses (15–18), MAPK signaling in the context of alphavirus infections has been only sparsely investigated. Only the activation of ERK in CHIKV-infected mouse astrocytes (19) and in SINV-infected human endothelial cells (20) has been described thus far. Here, we show that all three major branches of MAPK signaling were activated in the course of CHIKV infection.

Using a CHIKV replicon cell line in a high-throughput screen of approximately 3,000 compounds, we recently identified berberine, a relatively nontoxic isoquinoline alkaloid, as a potent inhibitor of CHIKV replication, with an additional effect on virus yield (21). Here, we sought to understand the mode of action of this compound and also addressed the question of its *in vivo* efficacy. We report for the first time the activation of the typical MAPK signaling pathways upon CHIKV infection and the collective impairment of these pathways upon treatment with berberine. Furthermore, we evaluated the efficacy of berberine in an *in vivo* mouse model and show the alleviation of CHIKV-induced inflammatory disease symptoms in this model upon treatment with berberine. Our results demonstrate the effectiveness of berberine as an antiviral drug against CHIKV and point toward MAPK signaling as a cellular target of berberine.

MATERIALS AND METHODS

Cell culture and inhibitors. Baby hamster kidney (BHK-21) cells and BSR T7/5 cells were cultured as described earlier (22, 23). Human osteosarcoma (HOS) cells, human embryonic kidney (HEK-293T; ATCC) cells and VeroE6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone), supplemented with 10% fetal bovine serum (FBS; HyClone). CRL-2522 (ATCC) human fibroblast cells were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin (Gibco). All cell lines were maintained at 37°C with 5% CO_2. C6/36 mosquito cells were cultured in Leibovitz L-15 medium (Gibco), supplemented with 10% FBS and maintained at 28°C with 5% CO_2. Berberine chloride (Sigma), abamectin (LKT Laboratories), ivermectin (Sigma), U0126 (Santa Cruz Biotechnology), SB203580 (Invivogen), and SP600125 (Invivogen) were dissolved in dimethyl sulfoxide (DMSO) and used at the indicated concentrations.

Viruses and virus infections. Wild-type (WT) CHIKV strain LR2006 OPY1 was rescued from the infectious clone SP6-ICRES (22). CHIKV-Rluc has a marker *Renilla reniformis* luciferase gene in the nsP3-coding region (22). Full-length CHIKV and ONNV infectious clones were produced using standard molecular biology techniques similar to those previously described (24). *Gaussia* luciferase (Gluc) from *Gaussia princeps* was inserted into CHIKV isolates from Reunion Island (LR2006 OPY1), Singapore (SGP11) (25), the Caribbean region (CNR20235) (26), and ONNV (27), at a site between the nonstructural and structural genes, together with an extra copy of the subgenomic promoter to ensure expression of the structural proteins. Similarly, CHIKV-LR2006 OPY1 was tagged with ZsGreen from *Anthozoa* reef coral. Virus stocks used for *in vitro* experiments were produced in BHK-21 or VeroE6 cells. For *in vivo* work, virus propagation was done in C6/36 cells, followed by purification and ultracentrifugation. Virus titers were determined by standard plaque assays in BHK-21 or VeroE6 cells, and virus stocks were stored at −80°C. Unless otherwise indicated, all experiments were performed with wild-type CHIKV LR2006 OPY1.

Mice. Animals were handled in strict accordance under the guidelines defined by the Agri-Food and Veterinary Authority (AVA) and National Advisory Committee for Laboratory Animal Research (NACLAR) of Singapore. Four-week-old C57BL6/J WT mice were bred and housed under specific-pathogen-free conditions at the Biological Resource Centre (BRC), Agency of Science, Technology and Research (A*STAR), Singapore. All procedures and experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC 151038).

Northern blotting. BHK-21 cells seeded on 6-well plates were infected with wild-type CHIKV under high multiplicity of infection (MOI) conditions (see the legend for Fig. 2 below). At 6 h postinfection (p.i.), cell culture supernatant was discarded and total RNA was purified using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Viral genomic and subgenomic RNAs were detected using a digoxigenin (DIG)-labeled RNA detection probe complementary to the 3′untranslated region (UTR) of the CHIKV genome as described earlier (28).

Cell cytotoxicity assays. Cell viability assays were carried out with the CellTiter-Glo (CTG) luminescent cell viability assay (Promega), which serves as a readout for cellular ATP levels, as previously described (29). Briefly, serial dilutions of berberine, at concentrations from 0.01 μM to 800 μM in triplicates, were added to HEK-293T, CRL-2522, or HOS cells seeded on 96-well plates. After 24 h (HEK-293T and CRL-2522) or 20 h (HOS), cell viability assays using CellTiter-Glo Reagent (Promega) were performed as per the manufacturer's protocol. After 1 h of incubation at room temperature, luminescent emissions were detected by the GloMax Multi-detection system (Promega) or Varioskan (Thermo Scientific). Additional cytotoxicity assays using the lactate dehydrogenase (LDH) cytotoxicity assay kit (Pierce Thermo Scientific) were performed in BHK-21 cells. The kit detects cellular LDH enzyme that has leaked into the supernatant due to loss of membrane integrity. Briefly, BHK-21 cells were seeded onto standard 96-well plates and treated with berberine at the same concentration range as described above. After 16 h, cell culture supernatant from the individual wells was transferred to a fresh 96-well plate, and the LDH assay was performed according to the manufacturer's instructions. Colorimetric absorbance from the enzyme substrate reaction was

measured at 490 nm, using the PerkinElmer EnSpire multimode plate reader. Signals were compared to mock-treated cells completely lysed with a lysis buffer provided with the kit.

Antiviral activity assays. Antiviral assays were performed similarly to the cell viability assays described above. Briefly, HEK-293T and CRL-2522 cells seeded on 96-well plates were infected with CHIKV-LR2006 OPY1-Gluc at an MOI of 0.1 and 1, respectively, in the presence of berberine at concentrations from 0.01 μM to 800 μM. HOS cells were infected with CHIKV-LR2006 OPY1-Rluc at an MOI of 1. For HEK-293T and CRL-2522, at 24 h p.i., 50 μl of conditioned medium from wells was transferred to a new semiwhite 96-well plate, followed by addition of 50 μl of 20 μM XenoLight RediJect coelenterazine h (PerkinElmer) diluted in phosphate-buffered saline (PBS) with 5 mM NaCl. Luminescent signals were immediately read by the GloMax Multi-detection system (Promega) at 1-s integration time. For HOS cells, at 20 h p.i., cell culture supernatants were discarded, cells were lysed, and luciferase activity was measured using the *Renilla* luciferase assay system (Promega) according to the manufacturer's instructions.

Plasmid constructs and transfection. The SFV *trans*-replication system polyprotein P123Z4 and template Tshort (template containing sequence encoding Rluc at the 5′ end) and Stluc (template containing sequence encoding Rluc under the subgenomic promoter) plasmids have been described elsewhere (23). CHIKV *trans*-replication system polyprotein P1234 and the T7-Rluc-Tom template (template containing sequence encoding Rluc at the 5′ end and the sequence encoding the red fluorescent protein Tomato under the subgenomic promoter) constructs have recently been described (28). Briefly, BSR T7/5 cells expressing bacteriophage T7 RNA polymerase seeded onto 96-well plates were transfected with the respective nonstructural polyprotein expression plasmids and plasmids encoding corresponding templates using LTX Lipofectamine according to the manufacturer's instructions and as previously described (23). At 4 h posttransfection, transfection medium was replaced with mock medium or with medium containing drugs at the indicated concentrations. At 16 h posttransfection, cells were lysed and luciferase values were measured as described above.

Human phosphokinase array. HOS cells seeded onto T-75 flasks were infected with wild-type CHIKV at an MOI of 10. Parallel flasks were mock infected without berberine, mock-infected with 5 μM berberine, or infected with CHIKV along with 5 μM berberine. After 1 h of adsorption, infection medium (minimal essential medium containing 0.2% bovine serum albumin [BSA] and 20 mM HEPES [pH 7.2]) was replaced with cell culture medium, also containing the same concentration of berberine for the respective flasks. At 8 h p.i., cells were lysed with lysis buffer provided with the Proteome Profiler Human Phospho-Kinase Array kit (R & D Systems). Samples were processed according to the manufacturer's instructions. Phosphoprotein spots were captured on an X-ray film, and their densities were quantified using ImageJ software.

SDS-PAGE and Western blotting. Cells in 6-well plates were lysed with 100 μl of 2× Laemmli buffer per well and solubilized and blotted as described earlier (21). Primary staining, using antibodies against CHIKV nsP1, nsP3, and capsid (all in-house), E2 proteins (a kind gift from Philippe Desprès, Insitut Pasteur), and mouse anti-actin (Sigma), and secondary staining were done as described earlier (21). Western blot evaluation of signaling was performed as described previously (7). The following antibodies were used according to the manufacturers' instructions: rabbit-anti-phospho-Akt (S473) (Cell Signaling number 4060), rabbit-anti-total-Akt (Cell Signaling number 4691), rabbit-anti-phospho-ERK1/2 (p44/42) (Cell Signaling number 4370), rabbit-anti-total-ERK1/2 (p44/42) (Cell Signaling number 4695), rabbit-anti-phospho-p38 MAPK (Cell Signaling number 4511), rabbit-anti-total-p38 MAPK (Cell Signaling number 8690), mouse-anti-phospho-SAPK/JNK (Cell Signaling number 9255), rabbit-anti-total-SAPK/JNK (Cell Signaling number 9252), goat anti-actin (Santa Cruz number 1616). All Western blot data are representative of at least two independent experiments.

Animal studies. Mice were subcutaneously inoculated with 10^6 PFU of CHIKV-LR2006 OPY1-ZsGreen in 30 μl PBS at the ventral side of the right hind footpad. On days 1 to 6 postinfection, mice were administered 10 mg/kg of body weight of berberine chloride diluted in PBS via intraperitoneal (i.p.) injection daily at 24-h intervals. Control mice were administered with PBS containing 4% DMSO (at concentrations similar to those of the berberine chloride preparations). Footpad joint swelling was determined daily from 0 to 14 days postinfection (dpi) by measuring the height and breadth of the right footpad using a digital caliper (Mitutoyo) and then quantified as the product of height and breadth. Disease score was expressed as the relative increase compared to preinfection (0 dpi) and was calculated as follows: $[(x - y)/y]$, where x represents the footpad measurement at the respective days postinfection and y is the measurement at 0 dpi. Ten microliters of blood from the tail vein was collected daily from 1 to 8 dpi and subsequently on every alternate day until 14 dpi to track for viremia (26, 30, 31).

Viral RNA extraction and RNAemia quantification. Ten microliters of blood collected from tail vein was diluted in 120 μl PBS and 10 μl citrate-phosphate-dextrose solution (Sigma-Aldrich). Viral RNAs were extracted using the QIAmp Viral RNA minikit (Qiagen) following the manufacturer's protocol. Quantification of CHIKV viral RNA copies with primers specific for nsP1 was performed using QuantiTect Probe reverse transcription-PCR (RT-PCR) (Qiagen) and the 7900HT Fast real-time PCR system machine (Applied Biosystems) under conditions described previously (32, 33).

Total RNA extraction and viral load quantification. Mice were euthanized, and joint footpad samples were removed and stored in TRIzol (Invitrogen) at −80°C. Tissues were homogenized using a rotor-stator homogenizer (Xiril Dispomix) at 5,000 rpm for 30 s. Homogenized tissues were mixed with 230 μl of chloroform and centrifuged at 13,000 rpm for 10 min at 4°C. The aqueous phase was collected, and total RNA was isolated using the RNeasy minikit (Qiagen) per the manufacturer's instructions. The concentration of extracted total RNA was determined with the NanoDrop 1000 Spectrophotometer (Thermo Scientific) and then diluted to 10 ng/μl. Diluted total RNA samples were then subjected to viral load quantification using primers specific for CHIKV E1 with QuantiTectProbe RT-PCR (Qiagen) and the 7900HT Fast real-time PCR system machine (Applied Biosystems), under conditions described previously (26).

Histology. Mice were euthanized, and joint footpad samples were harvested and stored in 10% neutral buffered formalin (Sigma-Aldrich). Fixed tissues were then decalcified and embedded in paraffin wax before 8-μm-thick sections were cut. Hematoxylin and eosin (H&E) staining was performed using established protocols as previously described (31, 33). HistoQuest software (TissueGnostics) was used for the quantification of cellular infiltration.

Data and statistical analyses. Half-maximal cytotoxic and effective concentrations (CC_{50} and EC_{50}) were determined from dose-response cell viability and antiviral curves, which were generated using OriginPro software. Statistical analyses were performed on GraphPad Prism 6.01 software and Microsoft Excel. For the titration experiments, the unpaired t test was used to compare drug-treated samples to negative-control DMSO samples. Mann-Whitney U test was used to compare *in vivo* PBS-treated and berberine-treated CHIKV-infected groups. P values of less than 0.05 are considered to be statistically significant.

RESULTS

Berberine's anti-CHIKV activity is not cell type specific. The data describing the *in vitro* efficacy of berberine against CHIKV were generated in BHK-21 cells with a complementary dose-response assay in Huh 7.5 cells (21). Both these cell lines are type I interferon (IFN) defective, allowing for rapid multiplication of the virus (34, 35). Here, we evaluated whether berberine possesses antiviral activity against CHIKV also in IFN-competent cells and employed a panel of human cell lines: HEK-293T, a cell line which

has been widely used in many studies with CHIKV (36, 37); the HOS cell line, suitable for analysis of CHIKV-induced signaling pathways (7); and the CRL-2522 human fibroblast cell line, since skin fibroblasts are considered to be the primary target cells for CHIKV replication in infected humans (38, 39). Dose-response assays for berberine were performed in these cell lines using luciferase-tagged versions of CHIKV strain LR2006 OPY1. Time points and infectious doses (for HEK-293T, 24 h at an MOI of 0.1; for HOS, 20 h at an MOI of 1; for CRL-2522, 24 h at an MOI of 1) were chosen based on luciferase signal optimization experiments done in these cell lines with the respective viruses. Cells were infected with the reporter virus in the presence of various concentrations of berberine ranging from 0.1 μM to 800 μM, and luciferase activity was measured as a readout for replication (Fig. 1A to C). In parallel, corresponding cell survival curves were generated in these cell types using the CTG assay, which measures cellular ATP levels from metabolically active cells. The EC_{50} was lowest in the HEK-293T cell line (4.5 μM) (Fig. 1A) with a selectivity index (CC_{50}/EC_{50}) of 45 (CC_{50} = 202.6 μM [Fig. 1D]). The EC_{50} in HOS cells was 12.2 μM (Fig. 1B; selectivity index = 35; CC_{50} = 429.5 μM [Fig. 1E]). In the CRL-2522 cells, an EC_{50} of 35.3 μM was determined (Fig. 1C). For this particular cell line, we repeatedly observed a lot of variation in the luciferase readouts at lower concentrations of berberine, probably a reason for the EC_{50} being higher than in the other two cell lines. Berberine was nontoxic in this cell line even at the highest concentration that was employed (800 μM), precluding determination of its selectivity index under these conditions (Fig. 1F). The high selectivity indices of berberine in these different human cell lines show that this antiviral is relatively nontoxic for human cells. Nevertheless, in order to verify the nontoxic nature of berberine, we performed an additional cell survival dose-response assay with the same concentration range of berberine in BHK-21 cells using a different cytotoxicity assay, the LDH assay, which assesses the membrane integrity of cells. In parallel, we also used the CTG assay measuring ATP levels, for comparison. The CC_{50} value for the CTG assay (533.2 μM; (Fig. 1G) was lower than that obtained with the LDH assay (1,545.4 μM; Fig. 1H), highlighting the sensitivity of the ATP detection cytotoxicity assay and showing that berberine is indeed nontoxic at the concentrations and under the conditions used.

Berberine is active against multiple CHIKV strains. Next, we employed the human fibroblast cell line CRL-2522 to analyze the effectiveness of berberine against different CHIKV strains. In addition to the LR2006 OPY1 strain used above, we used analogous reporter viruses from strain CHIKV-SGP11 (25), of the ECSA (East/Central/South African) genotype, as well as strain CNR-20235, which belongs to the Asian genotype and has been responsible for the outbreak in the Americas since late 2013 (26). In parallel, we also tested berberine against ONNV, the closest relative of CHIKV. A dose-dependent reduction in luciferase signals was measured for each of these viruses in response to berberine treatment (Fig. 2), with minor differences in the EC_{50}s for the different CHIKV strains: 37.6 μM, 44.2 μM, and 50.9 μM for LR2006 OPY1, SGP11, and CNR20235, respectively. The EC_{50} of berberine against ONNV was also in the same range (29.2 μM). Overall, the results in Fig. 2 indicate that berberine is a potent antiviral against multiple strains of CHIKV and likely several other alphaviruses in a variety of cell types.

Berberine retains its antiviral effect at high multiplicity of infection. Berberine had been shown to reduce CHIKV RNA synthesis, protein expression, and virus particle formation under low-MOI (0.01) conditions (21), where effects can be amplified over multiple replication cycles. To assess whether berberine is also antiviral against CHIKV in a high-MOI setting, we mock infected BHK-21 cells or infected them with wild-type CHIKV at an MOI of 10, either untreated or treated with 0.1% DMSO (solvent control) or berberine in a 2-fold dilution series ranging from 1.25 μM to 20 μM, present throughout the course of the infection. At 6 h postinfection (p.i.), supernatants were collected and subjected to conventional plaque assay titration on BHK-21 cells. Berberine lowered CHIKV titers in a dose-dependent manner with significant reduction at all concentrations down to 1.25 μM (Fig. 3A). Cell lysates from the corresponding wells were assessed for expression of viral nonstructural (nsP1 and nsP3) and structural (capsid and E2) proteins by Western blotting (Fig. 3B). Berberine proved to be efficient at reducing both viral nonstructural and structural protein levels under these high-MOI conditions. In an analogous setup, total RNA was isolated from infected cells, and viral genomic and subgenomic RNA levels were analyzed in a Northern blot assay (Fig. 3C). We also observed a dose-dependent reduction of viral RNA synthesis, evident at berberine concentrations above 5 μM. Taken together, these results show that berberine dose dependently reduced viral RNA and protein synthesis as well as viral titers under high-MOI conditions.

Berberine does not affect alphavirus entry or replication of template RNA in alphavirus *trans*-replication systems. We had earlier shown in a time-of-addition study that berberine affects the replication phase of the alphavirus life cycle with an additional effect on later steps as well (21). We ruled out any effect of berberine on virus entry: luciferase signals of a temperature-sensitive, luciferase-tagged Semliki Forest virus mutant (SFV-ts9) in an entry assay (described in reference 22) were not reduced upon berberine treatment (Fig. 4A). Results obtained using SFV might not necessarily apply to CHIKV, and therefore we performed an entry assay using CHIKV-Rluc at a high MOI of 50 for a short duration of 2 h and once again observed a similar result, whereby berberine treatment did not affect luciferase signals compared to known entry inhibitors such as chlorpromazine and bafilomycin A1 (Fig. 4B).

One possible explanation for the reduction in viral RNA and protein synthesis (Fig. 3) could be an inhibition of the replicase activity of the viral replication complex. In order to investigate this, we employed a *trans*-replication system, in which RNA replication and nonstructural protein expression are uncoupled by use of two separate plasmids, one encoding the viral replicase (nonstructural polyprotein) and one encoding reporter RNA as a template for the viral replicase, expressing *Renilla* luciferase. We used both the previously characterized SFV (23) and a recently developed CHIKV *trans*-replication system (28). In both of these systems, berberine had no effect on luciferase signals resulting from the replication of the independently provided template RNA by the viral replication machinery, which was in contrast to the two drugs abamectin (ABM) and ivermectin (IVM), which significantly reduced these signals (Fig. 4C and D). ABM and IVM had been identified as antivirals in our high-throughput screen and been shown to affect the early phase of virus replication (21). This result argues against a direct inhibition of viral replicase activity by berberine.

The alphavirus structural proteins are generated by translation of the subgenomic mRNA, transcribed from a promoter on the minus strand. We thus assessed if berberine's additional effect on

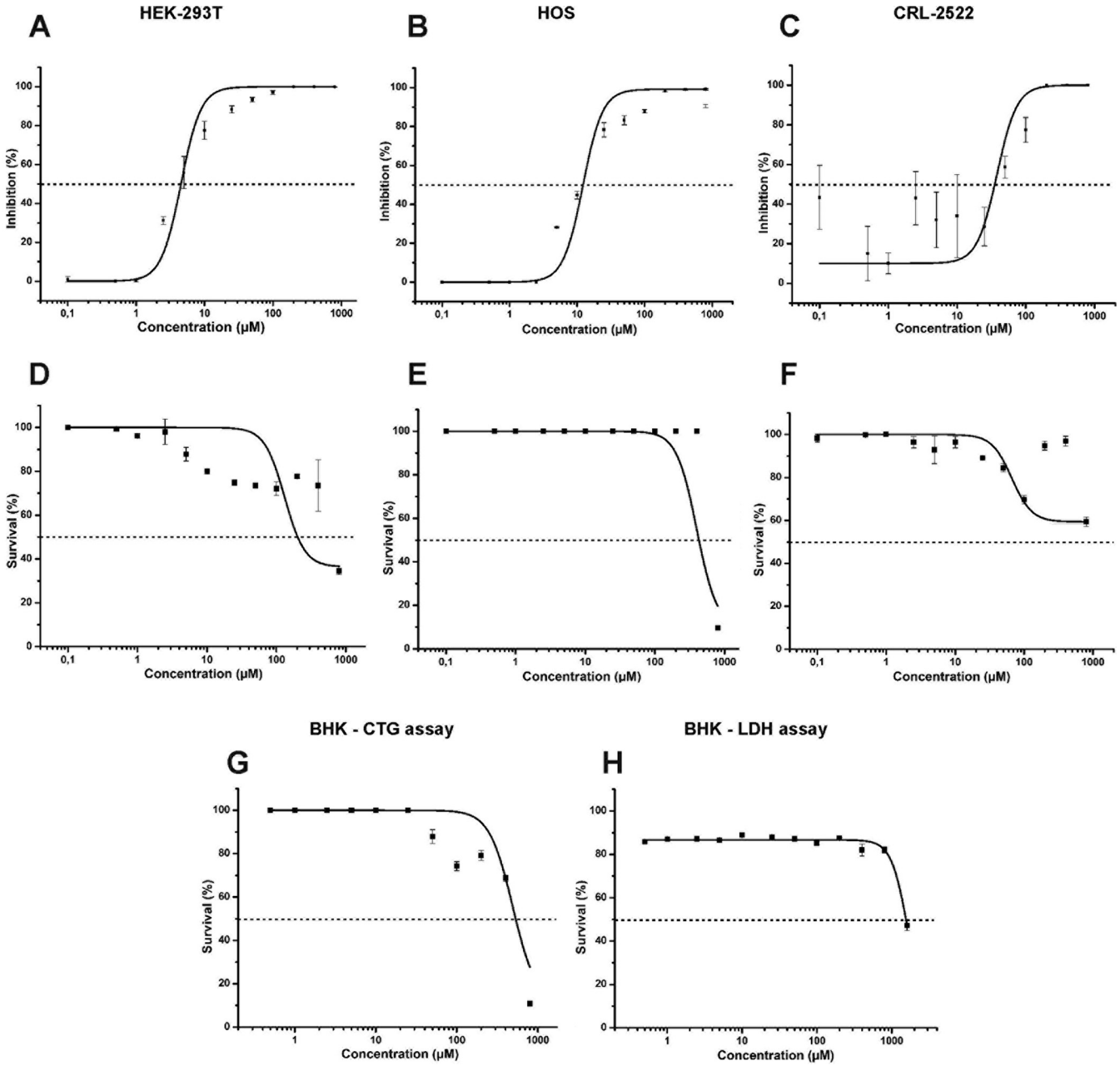

FIG 1 Berberine is antiviral and nontoxic in different human cell lines. (A to C) Dose-response assay to evaluate the antiviral activity of berberine in different human cell lines. (A) HEK-293T cells infected with Gluc-tagged CHIKV LR2006 OPY1 at an MOI of 0.1 for 24 h; (B) HOS cells infected with Rluc-tagged CHIKV LR2006 OPY1 at an MOI of 1 for 20 h; (C) CRL-2522 cells infected with Gluc-tagged CHIKV LR2006 OPY1 at an MOI of 1 for 24 h. After virus adsorption, infection medium was replaced with medium containing berberine at concentrations ranging from 0.01 μM to 800 μM. Percent inhibition values were calculated based on luciferase signals from infected untreated cells. (D to F) Cell survival dose-response curves to evaluate the cytotoxicity of berberine in human cell lines. Berberine treatment at concentrations ranging from 0.1 μM to 800 μM was given to HEK-293T cells for 24 h (D), HOS cells for 20 h (E), and CRL-2522 cells for 24 h (F). Percent survival values were calculated based on untreated mock-infected cells. (G and H) Additional cytotoxicity test to verify the nontoxic nature of berberine. BHK-21 cells were treated with medium containing berberine in the same concentration range, and cell survival was measured using the CTG (Cell Titer Glo) assay (G) or the LDH (lactate dehydrogenase) assay (H). Half-maximal (EC_{50}) and CC_{50} thresholds are marked with a dotted line. Assays were performed in triplicate wells. Data are presented as means ± standard errors of the means (SEM) ($n = 3$ for HEK-293T, $n = 2$ for HOS, $n = 2$ for CRL-2522, $n = 2$ for each assay in BHK-21 cells).

the later steps of the viral life cycle could be due to reduced protein expression from the subgenomic promoter. However, no significant effect of berberine on expression of luciferase under the control of the subgenomic promoter was observed. Again, ABM and IVM treatment clearly reduced these signals (Fig. 4E). These results indicate that berberine does not directly affect the alphavirus replicase activity. Hence, the drug may perturb alphavirus replication indirectly by affecting host components.

Berberine reduces CHIKV-induced MAPK signaling. Earlier studies have demonstrated that berberine impedes virus repli-

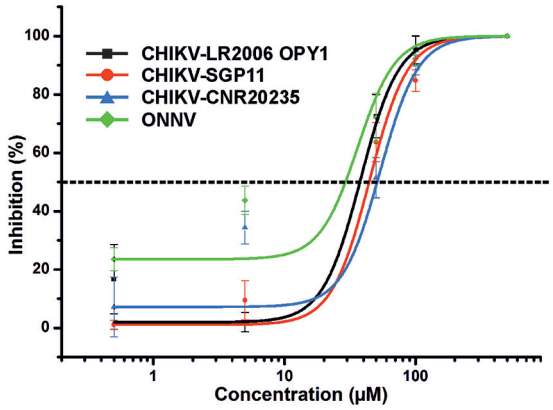

FIG 2 Demonstration of antiviral activity against multiple CHIKV strains and ONNV. CRL-2522 cells were infected with Gluc-tagged versions of CHIKV isolates LR2006 OPY1, SGP11, CNR20235, and ONNV at an MOI of 1. Berberine was added for 24 h, and Gluc activity was detected by luminescence signal. Percentage inhibition values were expressed relative to signals from untreated infected cells. Luciferase signals were measured from triplicate wells, and data are presented as means ± SEM ($n = 2$).

cation by modifying the cellular signaling environment (40–42). To pinpoint the pathways that are altered by berberine in CHIKV infection, we employed a human phosphokinase array that allows for detection of changes in the relative phosphorylation levels of a panel of different cellular kinases and their substrates. HOS cells were infected at an MOI of 10 for 8 h, a time when CHIKV nsP3 expression is readily detectable and virus-induced alteration of signaling pathways is seen (7). Berberine was employed at a concentration of 5 μM throughout the infection period. Four conditions were tested: mock infection or infection with CHIKV, each in the absence or presence, respectively, of berberine.

Results of the phosphokinase array showed that CHIKV infection in the absence of berberine led to more than 2-fold induction of the phosphorylation of each of the major MAP kinases: p38 MAPK, ERK, and JNK (Fig. 5A). All of these activations were clearly reduced on virus infection in the presence of berberine. Additionally, the phosphorylation of c-Jun, a transcription factor that is a substrate of both ERK and JNK (43), was induced almost 3-fold upon virus infection and comparatively reduced in virus-

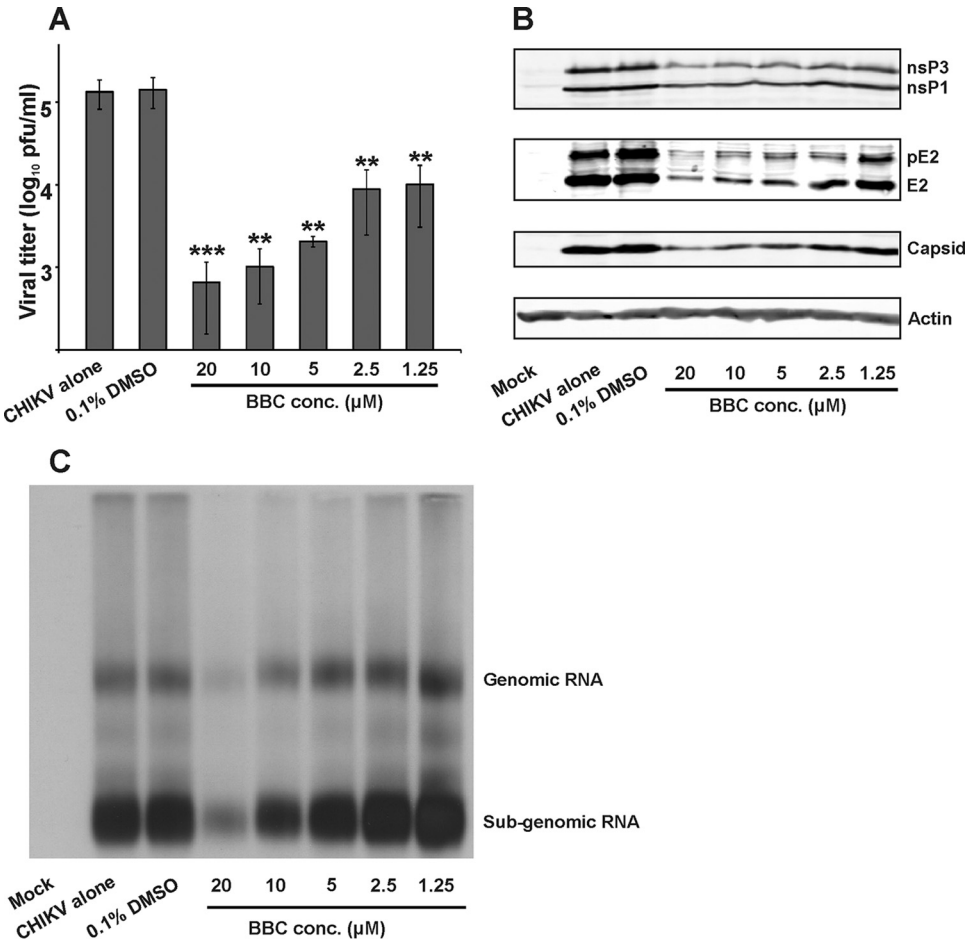

FIG 3 Berberine is effective even at high multiplicity of infection. BHK-21 cells were infected at an MOI of 10 with wild-type CHIKV for 6 h. Berberine (BBC) was present throughout the experiment in a 2-fold concentration series ranging from 20 μM to 1.25 μM. (A) Plaque assay viral titers from cell culture supernatants collected 6 h p.i. performed in triplicates (means from two independent experiments ± SEM). Statistical significance was determined using a one-way analysis of variance (ANOVA) test (***, $P < 0.001$; **, $P < 0.01$). (B) Cell lysates were analyzed by Western blotting for the presence of the indicated CHIKV proteins. Actin was used as a loading control. (C) Total RNA was isolated from parallel wells, and Northern blot analysis was done using probes specific to the 3′ untranslated region of the CHIKV genome, and the viral genomic and subgenomic RNAs were detected. Representative results from two independent experiments.

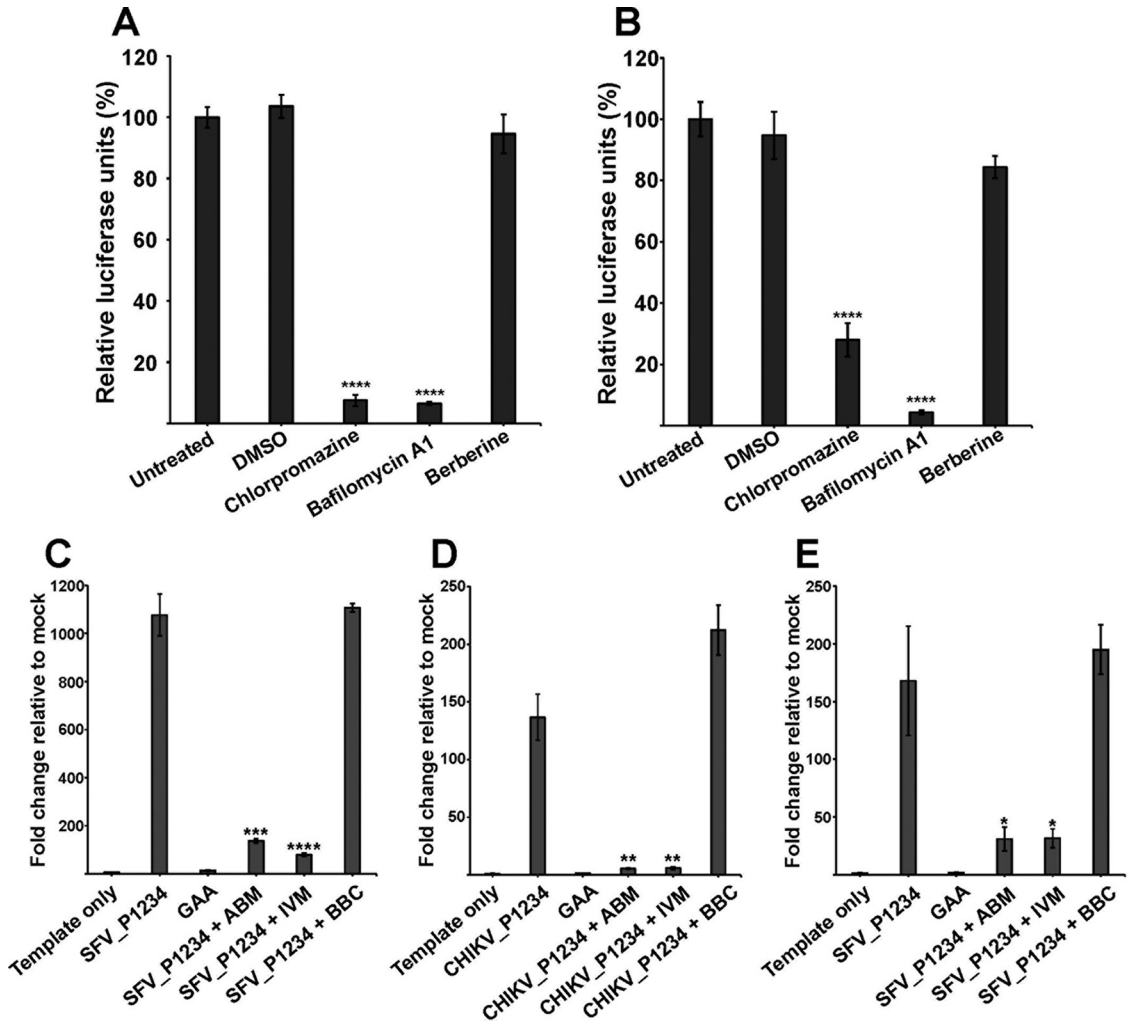

FIG 4 Berberine does not affect alphavirus entry or the replication of templates in *trans*-replication systems. (A and B) BHK-21 cells were infected with either SFV-ts9-Rluc (A) or CHIKV-Rluc (B) viruses at an MOI of 50 for 2 h at 39°C (A) or at 37°C (B). Infected cells were untreated or treated with 0.1% DMSO (solvent control), 50 μM chlorpromazine or 50 nM bafilomycin A1 (positive controls), or 3 μM berberine. At 2 h postinfection, cells were lysed and luciferase values were measured. Values are expressed as percent relative luciferase units compared to untreated infected samples. Luciferase signals were measured from quadruplicate wells, and data are presented as means ± SEM ($n = 2$). (C and D) Berberine was tested for its capacity to interfere with the enzymatic activities of the alphavirus *trans*-replication system. BSR T7/5 cells were transfected with SFV_P1234 polyprotein expression plasmid along with the Tshort template (*Renilla* luciferase at the 5′ end of the template) (C) or CHIKV_P1234 polyprotein expression plasmid along with the T7-Rluc-Tom template (*Renilla* luciferase at the 5′ end of the template and red fluorescent protein Tomato under the subgenomic promoter) (D). (E) Expression from the subgenomic promoter was measured in BSR T7/5 cells transfected with SFV_P1234 along with the Stluc template (*Renilla* luciferase under the subgenomic promoter). Transfection of only the template plasmid or transfection of template plasmid together with expression plasmid for P1234 harboring replication-defective polymerase (GDD to GAA mutation in nsP4) was used as negative control. At 4 h posttransfection, transfection medium was replaced with untreated cell culture medium or medium containing 3 μM abamectin (ABM), ivermectin (IVM), or berberine (BBC). Cells were lysed 16 h posttransfection, and luciferase signals were measured and expressed as fold changes relative to mock-transfected cells. Luciferase signals were measured from triplicate wells, and data are presented as means ± SEM ($n = 2$). Statistical significance was determined using a one-way ANOVA test (****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$).

infected, berberine-treated cells. Results shown here corroborated the CHIKV-induced phosphorylation of the Akt kinase (7), which was reduced to background levels by berberine. Furthermore, we observed a reduction in phosphorylation of the tumor suppressor p53 protein, one of the substrates of p38 MAPK (44), at three different residues upon CHIKV infection in the presence of berberine. Other factors altered by berberine treatment were the phosphorylation of the WNK1 ("with no K [lysine] protein kinase 1"), a substrate of Akt (45), and the expression of heat shock protein 60 (HSP60). None of the other proteins in the array was notably influenced by virus infection and/or berberine treatment.

This result was confirmed by immunoblots (Fig. 5B): we again observed the activation of all three major MAP kinases specifically by CHIKV infection and the subsequent reduction in their phosphorylation levels when berberine was present (Fig. 5B, compare lanes 3 and 4). CHIKV-induced Akt phosphorylation at Ser-473 was also reduced on berberine treatment, confirming the result from the phosphokinase array. Altogether, this set of results demonstrates that MAPK signaling pathways are robustly activated by CHIKV infection and that this activation is counteracted by berberine.

Effects of pharmacological inhibition of the MAP kinases on CHIKV replication. The effects of pharmacological inhibition of

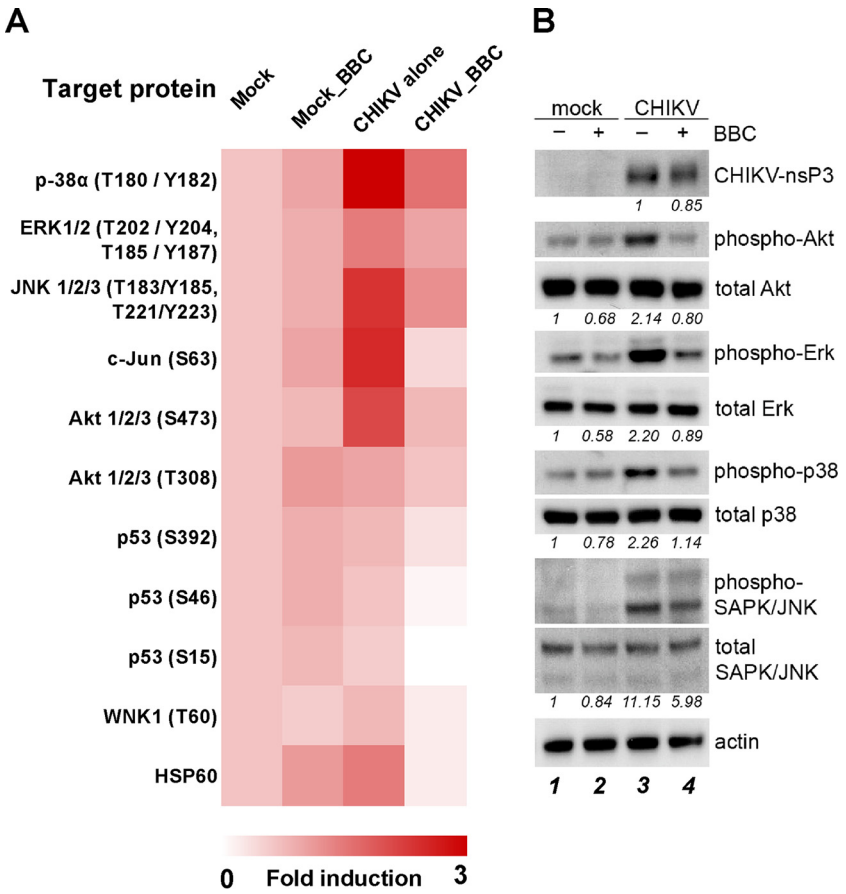

FIG 5 Berberine modulates CHIKV-induced signaling pathways. (A) Human phosphokinase array. HOS cells were either mock infected, mock infected with 5 μM berberine (Mock_BBC), infected with wild-type CHIKV (CHIKV alone), or infected with CHIKV in the presence of 5 μM BBC (CHIKV_BBC). Cells were lysed at 8 h p.i. and subjected to the array. Phosphoprotein spots were captured on an X-ray film, densitometric analysis was performed on ImageJ, and the relative phosphorylation levels were expressed as fold induction relative to untreated mock cells and represented as a heat map. The data are representative of two independent experiments. (B) Verification by immunoblotting. HOS cells were infected in the same pattern as in the phosphokinase array. Lysates were collected at 8 h p.i., followed by Western blotting for the indicated proteins. Densitometric data, which represent the mean ratio of phosphoprotein to the respective total protein signal, normalized to the mock-treated, mock-infected controls (infected mock-treated for nsP3), are indicated below the respective protein panels. Representative results from at least two independent experiments.

these kinases on CHIKV replication were then explored by known inhibitors: U0126, a specific inhibitor of MEK1/2, upstream of ERK (46); SB203580, a selective inhibitor of p38 MAPK (47); and SP600125, a potent and selective inhibitor of JNK (48) (Fig. 6A).

We first analyzed the effects of these inhibitors on CHIKV-mediated reporter protein expression. BHK-21 cells were left untreated or treated with 0.1% DMSO (solvent control) or with one of these inhibitors or berberine. Following pretreatment for 2 h, the cells were infected with CHIKV-Rluc at an MOI of 10 for another 6 h in the presence of the inhibitors (the experimental layout is shown in Fig. 6B), followed by cell lysis and luciferase activity measurement (Fig. 6C). Luciferase signals were significantly reduced by treatment with berberine, the p38 MAPK inhibitor SB203580, and the JNK inhibitor SP600125. Upstream inhibition of ERK by U0126 did not have any significant effect on CHIKV-mediated reporter expression. None of these inhibitor treatments significantly affected cell survival under the conditions employed (Fig. 6C).

Next, the effects of these inhibitors on viral titers were determined by infecting BHK-21 cells with wild-type CHIKV and measuring viral titers at 6 h p.i. by plaque assay (Fig. 6D). A large drop in viral titers (by nearly 3 log) was once again observed upon berberine treatment. Administration of the JNK inhibitor SP600125 yielded a reduction in viral titers by 2 log, consistent with the significant reduction of luciferase expression. However, the significant reduction in luciferase values upon treatment with the p38 MAPK inhibitor SB203580 was not reflected in a corresponding decrease in virus titer. Interestingly, the MEK inhibitor U0126, which did not reduce luciferase signals in the previous experiment, led to a significant reduction in viral titers by 1 log. This may imply a greater role of the ERK signaling cascade in virus egress than viral protein expression.

In a complementary experiment, the effects of these kinase inhibitors were assessed on CHIKV-induced MAPK activation by examining the phosphorylation status of their respective kinases under the exact same conditions using Western blotting (Fig. 6E). Berberine treatment once again showed reduced CHIKV-induced MAPK activation (compare lanes 5 and 6). Both U0126 and SP600125 completely abolished phosphorylation of the ERK and JNK proteins, respectively, in mock-infected and CHIKV-infected

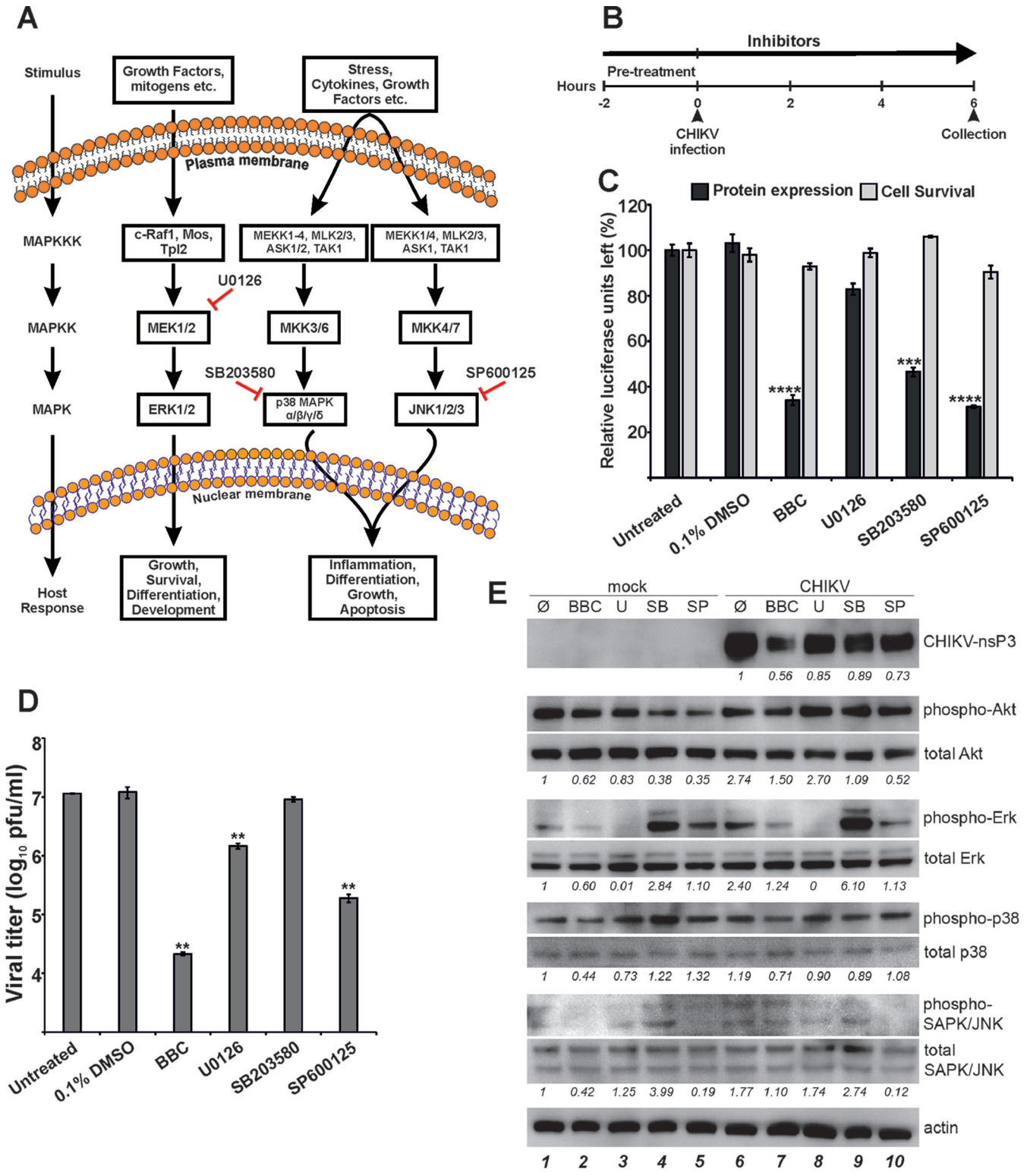

FIG 6 Effects of MAPK inhibitors on CHIKV. (A) Schematic diagram showing the three main groups of the MAPK signal transduction system: ERK, p38 MAPK, and JNK. Some of the main kinases at each stage are shown along with the inhibitors used in this study. (B) Experimental setup. BHK-21 cells were pretreated with berberine (5 μM), U0126 (20 μM), SB203580 (10 μM), or SP600125 (40 μM). DMSO (0.1%) was used as a solvent control. Following the pretreatment, cells were infected with wild-type or Rluc-tagged CHIKV at an MOI of 10 for 6 h, after which the respective samples were collected and processed. Inhibitors were present throughout the experiment. (C) Luciferase values derived from CHIKV-Rluc infection of BHK-21 cells in the experimental setup described above. Luciferase signals were measured from triplicate wells, and the data are presented as means ± SEM; representative results of two independent experiments. Statistical significance was determined by an unpaired *t* test (****, $P < 0.0001$; ***, $P < 0.001$). (D) Plaque assay titers performed in triplicates from BHK-21 cell culture supernatants infected with wild-type CHIKV in the layout described above. Means ± SEM from two independent experiments. Statistical significance was determined by an unpaired *t* test (***, $P < 0.001$). (E) Phosphorylation profiles of the respective kinases that were inhibited in this setup. Cell lysates from BHK-21 cells were collected, and Western blot analysis was performed for the indicated proteins (BBC, berberine; U, U0126; SB, SB203580; SP, SP600125). Densitometric data, which represent the mean ratio of phosphoprotein to the respective total protein signal, normalized to the mock-treated, mock-infected controls (infected mock-treated for nsP3), are indicated below the respective protein panels. Representative data set from at least two independent experiments.

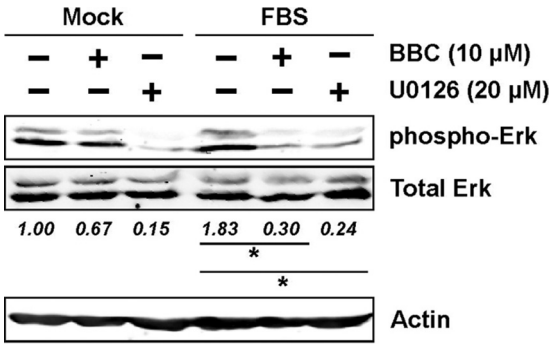

FIG 7 Berberine suppresses virus-independent ERK activation. HOS cells were starved in serum-free medium for 4 h and then treated either with fresh serum-free medium or with 100% FBS for 16 h with or without 10 μM berberine or 20 μM U0126. Densitometric data are the mean ratio of phosphoprotein to total protein signal, normalized to the loading control and mock-treated samples. Representative data from two independent experiments. Statistical significance was determined using a one-way ANOVA test (*, $P < 0.05$).

cells. SB203580, which is an inhibitor of p38 MAPK itself but does not prevent its phosphorylation by upstream kinases, did not affect levels of phospho-p38 but instead led to an increase in ERK and JNK phosphorylation.

Berberine is capable of impeding virus-independent ERK activation. The data described so far indicate that berberine reduces CHIKV-induced MAPK activation. Next, we investigated berberine's ability to suppress MAPK signaling independent of CHIKV infection. It is known that growth factors in serum can stimulate the ERK pathway (13); therefore, we performed an assay where HOS cells were starved in serum-free medium for 4 h, followed by addition of fresh medium devoid of serum or 100% FBS in the presence or absence of 10 μM berberine or 20 μM U0126 (positive control) for 16 h. We observed a marked increase of ERK activation in the FBS-treated samples compared to mock-treated ones. Addition of berberine significantly reduced the ERK activation in FBS-treated samples, similar to the positive control (Fig. 7). We did not observe any significant effect of berberine on the virus-independent activation of p38 MAPK and JNK (data not shown).

Berberine confers protection against CHIKV infection in mice. In order to fully recognize the potential of berberine as an antiviral drug and to ascertain the effectiveness of the drug *in vivo*, we explored the efficacy of berberine in the adult C57BL/6 mouse model (33). Four-week-old female C57BL6/J mice were infected with ZsGreen-tagged CHIKV-LR2006 OPY1, following which they were administered daily with either 10 mg/kg of berberine or PBS in equal volumes on days 1 to 6 postinfection. The frequency and duration of the treatment were selected so as to mimic a typical antiviral prescription and to determine if berberine was able to limit the symptoms of CHIKV-induced disease. Over the course of 14 days of infection, disease score (as a measure of footpad swelling) and RNAemia were monitored daily (Fig. 8A and B). When treated with berberine, mice displayed significantly reduced joint swelling on day 2 p.i. (Fig. 8A), mirrored in a lower viral load at the peak of RNAemia (Fig. 8B). RNAemia was observed to be constant from days 1 to 3 p.i., strongly indicating that berberine was able to control CHIKV replication. Importantly, berberine-treated mice showed significantly reduced joint inflammation at its peak on day 6 p.i. (Fig. 8A), to the extent that the swelling was reduced to almost baseline levels prior to infection, as seen in Fig. 8C. Quantification of virus load in the infected footpad at 6 dpi was determined using primers targeting the E1 region by reverse transcription-quantitative PCR (qRT-PCR) (Fig. 8D). At this late time point, when disease severity is no longer due to the effect of the virus, no significant difference was observed between the nontreated and berberine-treated groups (Fig. 8D). However, histological analysis performed on infected joint footpads harvested at 6 dpi clearly showed a lesser severity of inflammation and damage of the muscles, as well as a marked reduction in edema, in the berberine-treated mice (Fig. 8E). Quantification of cellular infiltration from H&E sections showed an overall lower percentage of infiltrates in the joints of berberine-treated than nontreated mice, indicating that the antiviral effects of berberine during the early phase of infection could also limit the infiltration of immune cells into the joints (Fig. 8F). Differences did not reach statistical significance due to experimental variation, although there was a trend of overall reduced inflammation in the berberine-treated mice. These results add value to berberine as an effective antiviral as well as an anti-inflammatory agent to abrogate joint inflammation. All berberine-treated mice survived and did not suffer from major weight loss, indicating that this dosage of the drug was well tolerated.

DISCUSSION

CHIKV has reemerged in the last decade, coinciding in part to its increased transmissibility in *Aedes albopictus* (49). The CHIKV epidemic continues to spread to the immunologically naive population in Central and South America (1, 50). In this scenario, therapeutic interventions are urgently needed to reduce the disease burden of the affected areas and restore the health of the afflicted individuals. The compound berberine was obtained and validated as a potential antiviral molecule in a screen of almost 3,000 bioactive molecules for antiviral activity against CHIKV (21). Berberine proved to affect a variety of circulating strains of CHIKV from both the ECSA and the Asian genotypes (Fig. 2). This might be particularly important in situations like the current CHIKV epidemic in Brazil, where strains belonging to different CHIKV genotypes were introduced at different times (51). Furthermore, berberine displayed similar activity against the related alphavirus ONNV and is thus likely to be broadly effective to treat alphaviral disease in general (21).

Here we endeavored to characterize berberine's inhibitory mechanism. We show that berberine reduced viral RNA synthesis, protein expression, and titers in several cell lines and at a high MOI in a dose-dependent manner. Interestingly, we did not observe a decrease in viral RNA synthesis at lower concentrations that were sufficient to reduce viral protein expression and virus titers (Fig. 3). Berberine did not markedly affect either virus entry (Fig. 4A and B) or the enzymatic activity of the viral replicase. The latter was found in a *trans*-replication system, an approach that had been used before to evaluate the direct effect of potential antivirals on the viral replicase (52). While berberine did not have any significant influence on replication in this system, other antiviral compounds (ABM and IVM), obtained in our earlier screen, significantly reduced the luciferase readout (Fig. 4C to E). Thus, these two compounds are likely to target elements in the viral life cycle other than berberine. The result that berberine treatment did not affect replicase activity but dose dependently lowered viral

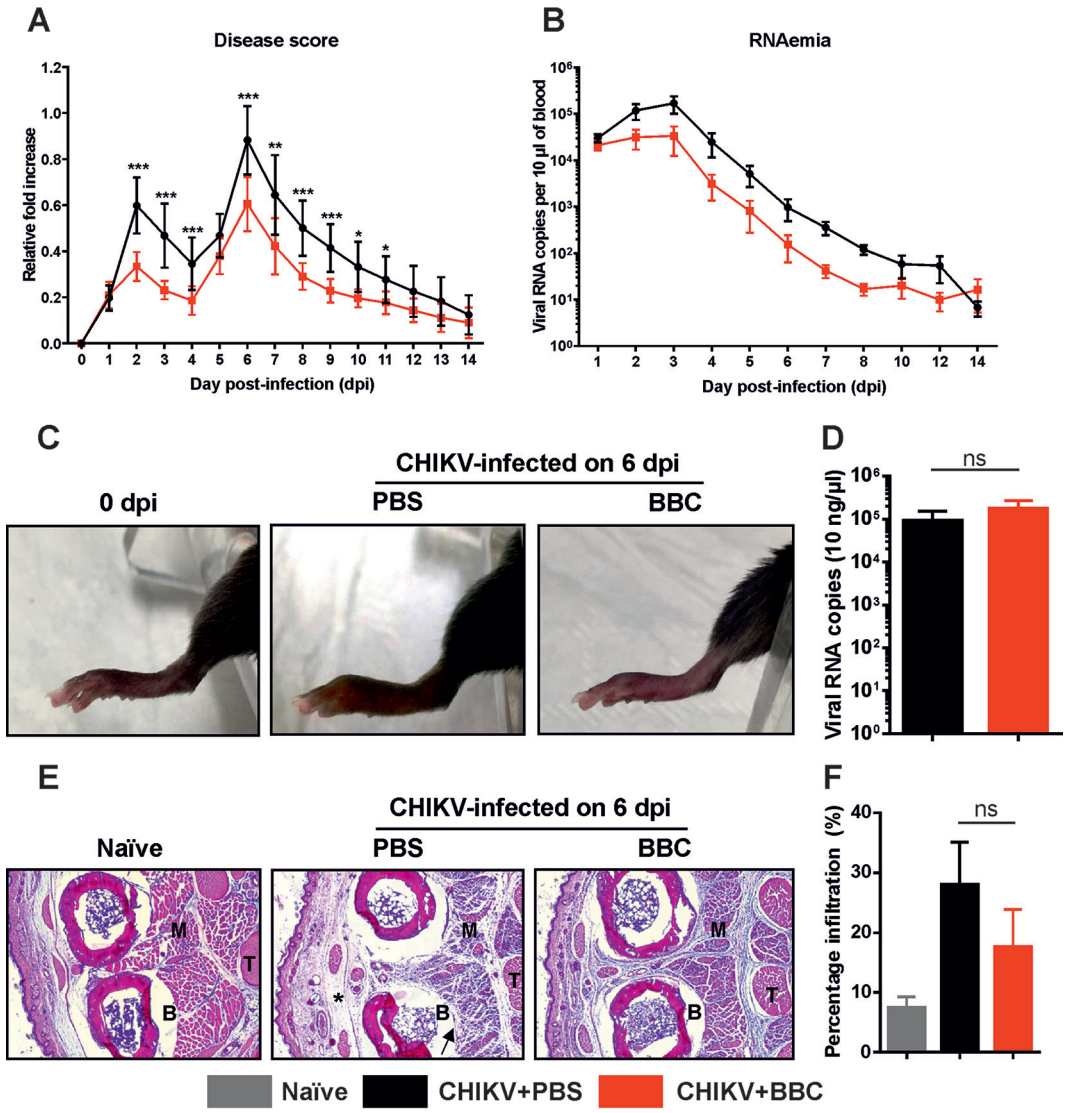

FIG 8 Berberine treatment ameliorates joint swelling in CHIKV-infected mice. Four-week-old C57BL6/J wild-type mice ($n = 8$ to 12 per group) were injected via i.p. with PBS or 10 mg/kg of berberine (BBC) daily on days 1 to 6 post-CHIKV infection after subcutaneous (s.c.) inoculation of 1 million PFU of ZsG-tagged CHIKV-LR2006 OPY1 at the hind limb footpad. (A) Joint footpad swelling was measured daily for disease score from 1 to 14 dpi. Data are presented as means ± standard deviations (SD) and representative of three independent experiments. (B) RNAemia was determined from blood collected from tail vein from 1 to 14 dpi. Data are presented as means ± SEM and representative of three independent experiments. (C) Representative images of joint footpad before and after CHIKV infection on days 0 and 6 p.i. (D) Viral load in the infected footpad ($n = 8$ per group) was analyzed. Data are presented as means ± SD. (E) Histological analysis at 6 days p.i. of of joint footpad samples from naive and CHIKV-infected mice treated with either PBS or BBC and stained with H&E. *, edema; arrow, infiltrates; B, bone; M, muscle; T, tendon. Images are representative of two mice per group at a magnification of ×4. (F) Percentage infiltration of cells at 6 days p.i. was quantified as the percentage of area of infiltrates to the total area from images taken ($n = 4$) at a magnification of ×10 using HistoQuest. Statistical analysis for all data was carried out using the Mann-Whitney two-tailed test (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$).

RNA and protein levels points toward an effect on one or more host components induced by virus infection, but not by the *trans*-replication system.

We therefore analyzed how berberine specifically modifies the cellular signaling environment to hamper CHIKV replication and found that all three major MAPK signaling pathways, ERK, p38 MAPK, and JNK, as well as the PI3K-Akt pathway were activated upon infection with CHIKV and reduced upon treatment of infected cells with berberine (Fig. 5A and B). This is to our knowledge the first experimental evidence of all major MAPK signaling pathways being concomitantly activated in the course of CHIKV infection in human cells. The induction of the Ras-Raf-MEK-ERK pathway has been shown for different RNA viruses like hepatitis C virus (53) and dengue virus (54) and also alphaviruses, namely, Sindbis virus (SINV) (20) and Venezuelan equine encephalitis virus (VEEV) (55). This pathway appears to be activated by these different viruses, potentially to confer a prosurvival status to the infected cells in order to maintain a productive infection cycle and generate sufficient progeny virions. CHIKV also activates this pathway and has in addition been shown to induce other prosurvival signaling cascades like the PI3K-Akt (7) and autophagy (8), likely to maximize its replicative potential in the infected host

cells. Our experiments showed a reduction in viral titers by approximately 1 log upon inhibition of the ERK axis with U0126 (Fig. 6D). The result that U0126 did not significantly affect virus-mediated protein expression in the luciferase assay but resulted in a marked reduction in viral titers suggests a role of the ERK pathway in virus maturation or egress. The potential relevance and mechanism of ERK signaling for virus assembly/budding remain to be determined. Recently, it was shown that ERK inhibition by Ag-126 and other MEK1/2 inhibitors reduced the titers of the alphavirus VEEV at an MOI of 0.1, whereas U0126 did not have any significant effect in that study (55). Our results, on the other hand, showed a 1-log reduction in CHIKV titers by U0126 in high-MOI experiments, highlighting the importance of this signaling cascade in the CHIKV life cycle.

The other two branches of MAPK signaling, p38 MAPK and JNK, were also robustly activated upon infection with CHIKV both in HOS and in BHK-21 cells. This has been shown for many RNA viruses, including the alphavirus SINV, which was reported to induce the activation of p38 MAPK and also the phosphorylation of HSP27 (heat shock protein 27), one of its substrates (56). For Japanese encephalitis virus (JEV), the unfolded protein response (UPR), triggered by excess viral protein synthesis in the endoplasmic reticulum, results in eventual apoptosis, mediated in a p38 MAPK-dependent manner (57). Recently, the IRE-1/JNK pathway stemming from the UPR was also implicated in JEV-induced apoptosis (58). CHIKV (59) and SFV (60) induce the UPR, through the synthesis of the structural glycoproteins in the ER lumen, and this might be one possible mechanism for alphavirus-induced p38 MAPK and JNK signaling, which would not occur in the *trans*-replication system. Here, we show that CHIKV infection activated the p38 MAPK and JNK pathways (Fig. 5). Inhibitors of these kinases led to a significant reduction in viral protein expression (Fig. 6C). On analysis of the protein phosphorylation profiles under these conditions, we observed that the p38 MAPK inhibitor SB203580 led to increased phosphorylation of ERK and JNK (Fig. 6E). There have been reports that inhibition of basal p38 activity may result in increased ERK activation due to cross talk between these pathways (61, 62). One of these studies also reported the detection of an SB203580 metabolite in human hepatocytes that may result in increased activation of JNK (62). This might be why no effect on viral titers was observed with SB203580 (Fig. 6D), due to such a compensatory boost in ERK and JNK levels. On the other hand, administration of the JNK inhibitor SP600125, where such compensatory activation of other MAPK pathways was not evident, reduced viral titers significantly (Fig. 6D).

Taken together, pharmacological intervention with each of the three CHIKV-induced branches of MAPK signaling led to a clear drop in viral protein expression and/or viral titers. This implies that the activation of MAPK signaling upon CHIKV infection has proviral functions. It remains unclear which of the many downstream targets of these MAPK signaling pathways is particularly relevant for virus replication and maturation. The specific molecular mechanism of CHIKV-induced MAPK and also Akt activation remains unresolved, but these activations may be interconnected, as there is evidence of cross talk between these pathways (63).

In CHIKV-infected cells, berberine lowered the activation of all three major MAPK signaling pathways and drastically reduced viral protein expression and titers at the same time, more than upon administration of either of the specific inhibitors of ERK, p38, or JNK signaling. Thus, berberine led to a combined reduction of CHIKV-induced MAPK signaling in general. Berberine strongly reduced ERK activation also independently of CHIKV infection, indicating the ERK axis as a molecular target of the drug (Fig. 7). It is hence conceivable that berberine predominantly targets the ERK arm of MAPK signaling and thereby reduces CHIKV replication, in consequence leading to an overall decrease in virus-induced MAPK signaling. Thus, the reduction of CHIKV-induced MAPK activation is likely to be an effect of berberine on host signaling factors rather than a virus-specific process being targeted that leads to diminished signaling. While the specific molecular target of berberine remains unknown, we think that the drug may affect processes upstream of the final effector MAP kinases. It appears likely that berberine has pleiotropic molecular targets rather than affecting one specific cellular factor. This could explain the low toxicity of the compound and the combined dampening of multiple, partially interconnected signaling pathways. Reduction in viral protein expression and titers by berberine is likely to be a consequence of a decrease in virus-induced signaling. We cannot exclude that other pathways and molecular features are affected by berberine and contribute to its antiviral capacity.

Finally, we tested berberine in a mouse model that recapitulates viremia and arthritic manifestations of CHIKV disease. In wild-type C57BL6/J mice, we show that berberine was effective at reducing the viral load in infected mice when administered starting 1 day after the infection (Fig. 8B). We also found that berberine was able to significantly decrease the second peak of joint swelling at 6 dpi, by limiting the infiltration of immune cells and reducing the extent of vascular leakage or edema (Fig. 8A, C, E, and F). The protective effect of berberine can also be extended to the muscle regions of the footpad, as it was clearly shown to reduce myositis (Fig. 8C and E). Virus loads in the infected joints at 6 dpi showed no difference between the two groups, suggesting that berberine acts as an early antiviral, controlling the viral load in the early phase of the disease, and no longer has an effect at the later time point of 6 dpi (Fig. 8D). Nevertheless, we cannot exclude that the disease-reducing effect of berberine *in vivo* is in part due to its anti-inflammatory properties, in addition to the inhibition of virus-induced MAPK signaling as observed *in vitro*. The berberine dosage of 10 mg/kg given to the mice is well below the 50% lethal dose (LD_{50}) of 58 mg/kg (i.p.) as reported by Kheir and colleagues (64). Taken together, the *in vivo* results show dual roles of berberine as an antiviral and an anti-inflammatory agent, and this is one of the few reports of an antiviral against CHIKV showing efficacy in a mouse model (3). Earlier, favipiravir was shown to protect against neurological complications and death in a mouse model of lethal CHIKV infection, which does not represent disease outcomes in the majority of CHIKV-infected patients (65). Interestingly, treatments were given either 24 h before infection or 4 h p.i., whereas in our study, berberine treatment was started only at 1 dpi. Start of treatment at 1 dpi is a realistic time frame for postexposure therapy, further supporting the potential of berberine as a good therapeutic option for chikungunya fever and likely other alphaviral diseases. Bindarit, an inhibitor of monocyte chemotactic protein synthesis, is the only other compound described to reduce CHIKV-induced arthritis in a mouse model similar to the one used in our study (66).

Based on our results obtained in cell culture systems, we believe that this alleviation of disease by berberine treatment is at least in

part due to the lowered activation of MAPK signaling. Indeed, berberine was reported to reduce the activation of signaling pathways like p38 MAPK and JNK in osteoarthritis synovial fibroblasts as well as cartilage destruction in a rat model of osteoarthritis (67), pointing to its anti-inflammatory properties. Further research is warranted to define the effects of berberine on CHIKV inflammatory disease markers *in vivo*. Our data strongly indicate that berberine is worth pursuing and optimizing as a therapeutic compound against chikungunya fever. Berberine clearly reduced CHIKV-induced MAPK signaling, relevant for the viral life cycle, a result that suggests that the antiviral activity of berberine can at least in part be explained by its effect on these virus-activated signaling cascades. In addition, our results argue in favor of further research to explore the possibility of repurposing approved drugs or compounds in clinical trials that target MAPK signaling as potential antivirals against CHIKV and other alphaviruses.

ACKNOWLEDGMENTS

We thank Philippe Desprès (Institut Pasteur) for anti-CHIKV-E2 monoclonal antibodies and Bhupendra Verma (University of Helsinki) for the SB203580 and SP600125 inhibitors. We acknowledge the Drug Discovery and Chemical Biology Network funded by Biocenter Finland, for providing access to screening instrumentation. We are also grateful to Mutant Mouse Collection (SIgN, A*STAR) and A*STAR Biological Resource Centre (BRC) for their assistance with breeding and supply of mice used in this study, and Advanced Molecular Pathology Laboratory (A*STAR) for their histology services. We declare that we have no competing interests.

This work was funded by the Academy of Finland (grant 265997 to T.A.), Biomedical Research Council, A*STAR (to L.F.P.N.), the Swedish Cancer Foundation (CAN2012/789 to G.M.M.), the Swedish Research Council (621-2014-4718 to G.M.M.), the Estonian Ministry of Education and Research (IUT20-27 to A.M.), and the German Research Foundation (TH1896/1-1 and TH1896/3 to B.T.). F.S.V. is a fellow of the Integrative Life Sciences doctoral program. The funders had no role in the experimental design, data analysis and interpretation of the results or the decision to submit this work for publication.

FUNDING INFORMATION

This work, including the efforts of Tero Ahola, was funded by Academy of Finland (265997). This work, including the efforts of Lisa F. P. Ng, was funded by Biomedical Research Council, A*STAR. This work, including the efforts of Gerald Michael McInerney, was funded by Swedish Cancer Foundation (CAN2012/789). This work, including the efforts of Gerald Michael McInerney, was funded by Swedish Research Council (621-2014-4718). This work, including the efforts of Andres Merits, was funded by Estonian Ministry for Education and Research (IUT20-27). This work, including the efforts of Bastian Thaa, was funded by German Research Foundation (TH1896/1-1). This work, including the efforts of Bastian Thaa, was funded by German Research Foundation (TH1896/3).

The funders had no role in experimental design, data analysis and interpretation of the results, or the decision to submit this work for publication.

REFERENCES

1. **Weaver SC, Lecuit M.** 2015. Chikungunya virus and the global spread of a mosquito-borne disease. N Engl J Med **372:**1231–1239. http://dx.doi.org/10.1056/NEJMra1406035.
2. **Weaver SC, Forrester NL.** 2015. Chikungunya: evolutionary history and recent epidemic spread. Antiviral Res **120:**32–39. http://dx.doi.org/10.1016/j.antiviral.2015.04.016.
3. **Abdelnabi R, Neyts J, Delang L.** 2015. Towards antivirals against chikungunya virus. Antiviral Res **121:**59–68. http://dx.doi.org/10.1016/j.antiviral.2015.06.017.
4. **Ahola T, Courderc T, Ng LF, Hallengard D, Powers A, Lecuit M, Esteban M, Merits A, Roques P, Liljestrom P.** 2015. Therapeutics and vaccines against chikungunya virus. Vector Borne Zoonotic Dis **15:**250–257. http://dx.doi.org/10.1089/vbz.2014.1681.
5. **Gould EA, Coutard B, Malet H, Morin B, Jamal S, Weaver S, Gorbalenya A, Moureau G, Baronti C, Delogu I, Forrester N, Khasnatinov M, Gritsun T, de Lamballerie X, Canard B.** 2010. Understanding the alphaviruses: recent research on important emerging pathogens and progress towards their control. Antiviral Res **87:**111–124. http://dx.doi.org/10.1016/j.antiviral.2009.07.007.
6. **Rupp JC, Sokoloski KJ, Gebhart NN, Hardy RW.** 2015. Alphavirus RNA synthesis and non-structural protein functions. J Gen Virol **96:**2483–2500. http://dx.doi.org/10.1099/jgv.0.000249.
7. **Thaa B, Biasiotto R, Eng K, Neuvonen M, Gotte B, Rheinemann L, Mutso M, Utt A, Varghese F, Balistreri G, Merits A, Ahola T, McInerney GM.** 2015. Differential phosphatidylinositol-3-kinase-Akt-mTOR activation by Semliki Forest and chikungunya viruses is dependent on nsP3 and connected to replication complex internalization. J Virol **89:**11420–11437. http://dx.doi.org/10.1128/JVI.01579-15.
8. **Joubert PE, Werneke SW, de la Calle C, Guivel-Benhassine F, Giodini A, Peduto L, Levine B, Schwartz O, Lenschow DJ, Albert ML.** 2012. Chikungunya virus-induced autophagy delays caspase-dependent cell death. J Exp Med **209:**1029–1047. http://dx.doi.org/10.1084/jem.20110996.
9. **Schilte C, Couderc T, Chretien F, Sourisseau M, Gangneux N, Guivel-Benhassine F, Kraxner A, Tschopp J, Higgs S, Michault A, Arenzana-Seisdedos F, Colonna M, Peduto L, Schwartz O, Lecuit M, Albert ML.** 2010. Type I IFN controls chikungunya virus via its action on nonhematopoietic cells. J Exp Med **207:**429–442. http://dx.doi.org/10.1084/jem.20090851.
10. **White LK, Sali T, Alvarado D, Gatti E, Pierre P, Streblow D, Defilippis VR.** 2011. Chikungunya virus induces IPS-1-dependent innate immune activation and protein kinase R-independent translational shutoff. J Virol **85:**606–620. http://dx.doi.org/10.1128/JVI.00767-10.
11. **Morrison DK.** 2012. MAP kinase pathways. Cold Spring Harb Perspect Biol **4**(11):pii:a011254. http://dx.doi.org/10.1101/cshperspect.a011254.
12. **Cargnello M, Roux PP.** 2011. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev **75:**50–83. http://dx.doi.org/10.1128/MMBR.00031-10.
13. **Yoon S, Seger R.** 2006. The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. Growth Factors **24:**21–44. http://dx.doi.org/10.1080/02699050500284218.
14. **Darling NJ, Cook SJ.** 2014. The role of MAPK signalling pathways in the response to endoplasmic reticulum stress. Biochim Biophys Acta **1843:**2150–2163. http://dx.doi.org/10.1016/j.bbamcr.2014.01.009.
15. **Borgeling Y, Schmolke M, Viemann D, Nordhoff C, Roth J, Ludwig S.** 2014. Inhibition of p38 mitogen-activated protein kinase impairs influenza virus-induced primary and secondary host gene responses and protects mice from lethal H5N1 infection. J Biol Chem **289:**13–27. http://dx.doi.org/10.1074/jbc.M113.469239.
16. **Ludwig S, Ehrhardt C, Neumeier ER, Kracht M, Rapp UR, Pleschka S.** 2001. Influenza virus-induced AP-1-dependent gene expression requires activation of the JNK signaling pathway. J Biol Chem **276:**10990–10998. http://dx.doi.org/10.1074/jbc.M009902200.
17. **Pleschka S.** 2008. RNA viruses and the mitogenic Raf/MEK/ERK signal transduction cascade. Biol Chem **389:**1273–1282.
18. **Qadri I, Iwahashi M, Capasso JM, Hopken MW, Flores S, Schaack J, Simon FR.** 2004. Induced oxidative stress and activated expression of manganese superoxide dismutase during hepatitis C virus replication: role of JNK, p38 MAPK and AP-1. Biochem J **378:**919–928. http://dx.doi.org/10.1042/bj20031587.
19. **Das T, Hoarau JJ, Jaffar Bandjee MC, Maquart M, Gasque P.** 2015. Multifaceted innate immune responses engaged by astrocytes, microglia and resident dendritic cells against Chikungunya neuroinfection. J Gen Virol **96:**294–310. http://dx.doi.org/10.1099/vir.0.071175-0.
20. **Rust NM, Papa MP, Scovino AM, da Silva MM, Calzavara-Silva CE, Marques ET, Jr, Pecanha LM, Scharfstein J, Arruda LB.** 2012. Bradykinin enhances Sindbis virus infection in human brain microvascular endothelial cells. Virology **422:**81–91. http://dx.doi.org/10.1016/j.virol.2011.10.003.
21. **Varghese FS, Kaukinen P, Glasker S, Bespalov M, Hanski L, Wennerberg K, Kummerer BM, Ahola T.** 2016. Discovery of berberine, abamectin and ivermectin as antivirals against chikungunya and other alphaviruses. Antiviral Res **126:**117–124. http://dx.doi.org/10.1016/j.antiviral.2015.12.012.

22. **Pohjala L, Utt A, Varjak M, Lulla A, Merits A, Ahola T, Tammela P.** 2011. Inhibitors of alphavirus entry and replication identified with a stable Chikungunya replicon cell line and virus-based assays. PLoS One **6:**e28923. http://dx.doi.org/10.1371/journal.pone.0028923.
23. **Spuul P, Balistreri G, Hellstrom K, Golubtsov AV, Jokitalo E, Ahola T.** 2011. Assembly of alphavirus replication complexes from RNA and protein components in a novel trans-replication system in mammalian cells. J Virol **85:**4739–4751. http://dx.doi.org/10.1128/JVI.00085-11.
24. **Tsetsarkin K, Higgs S, McGee CE, De Lamballerie X, Charrel RN, Vanlandingham DL.** 2006. Infectious clones of Chikungunya virus (La Reunion isolate) for vector competence studies. Vector Borne Zoonotic Dis **6:**325–337. http://dx.doi.org/10.1089/vbz.2006.6.325.
25. **Her Z, Malleret B, Chan M, Ong EK, Wong SC, Kwek DJ, Tolou H, Lin RT, Tambyah PA, Renia L, Ng LF.** 2010. Active infection of human blood monocytes by Chikungunya virus triggers an innate immune response. J Immunol **184:**5903–5913. http://dx.doi.org/10.4049/jimmunol.0904181.
26. **Teo TH, Her Z, Tan JJ, Lum FM, Lee WW, Chan YH, Ong RY, Kam YW, Leparc-Goffart I, Gallian P, Renia L, de Lamballerie X, Ng LF.** 2015. Caribbean and La Reunion Chikungunya virus isolates differ in their capacity to induce proinflammatory Th1 and NK cell responses and acute joint pathology. J Virol **89:**7955–7969. http://dx.doi.org/10.1128/JVI.00909-15.
27. **Bessaud M, Peyrefitte CN, Pastorino BA, Gravier P, Tock F, Boete F, Tolou HJ, Grandadam M.** 2006. O'nyong-nyong virus, Chad. Emerg Infect Dis **12:**1248–1250. http://dx.doi.org/10.3201/eid1208.060199.
28. **Utt A, Quirin T, Saul S, Hellstrom K, Ahola T, Merits A.** 2016. Versatile trans-replication systems for Chikungunya virus allow functional analysis and tagging of every replicase protein. PLoS One **11:**e0151616. http://dx.doi.org/10.1371/journal.pone.0151616.
29. **Ching KC, Kam YW, Merits A, Ng LF, Chai CL.** 2015. Trisubstituted thieno[3,2-b]pyrrole 5-carboxamides as potent inhibitors of alphaviruses. J Med Chem **58:**9196–9213. http://dx.doi.org/10.1021/acs.jmedchem.5b01047.
30. **Lee WW, Teo TH, Her Z, Lum FM, Kam YW, Haase D, Renia L, Rotzschke O, Ng LF.** 2015. Expanding regulatory T cells alleviates chikungunya virus-induced pathology in mice. J Virol http://dx.doi.org/10.1128/JVI.00998-15.
31. **Teng TS, Foo SS, Simamarta D, Lum FM, Teo TH, Lulla A, Yeo NK, Koh EG, Chow A, Leo YS, Merits A, Chin KC, Ng LF.** 2012. Viperin restricts chikungunya virus replication and pathology. J Clin Invest **122:**4447–4460. http://dx.doi.org/10.1172/JCI63120.
32. **Kam YW, Lum FM, Teo TH, Lee WW, Simarmata D, Harjanto S, Chua CL, Chan YF, Wee JK, Chow A, Lin RT, Leo YS, Le Grand R, Sam IC, Tong JC, Roques P, Wiesmuller KH, Renia L, Rotzschke O, Ng LF.** 2012. Early neutralizing IgG response to Chikungunya virus in infected patients targets a dominant linear epitope on the E2 glycoprotein. EMBO Mol Med **4:**330–343. http://dx.doi.org/10.1002/emmm.201200213.
33. **Teo TH, Lum FM, Claser C, Lulla V, Lulla A, Merits A, Renia L, Ng LF.** 2013. A pathogenic role for CD4+ T cells during Chikungunya virus infection in mice. J Immunol **190:**259–269. http://dx.doi.org/10.4049/jimmunol.1202177.
34. **Breakwell L, Dosenovic P, Karlsson Hedestam GB, D'Amato M, Liljestrom P, Fazakerley J, McInerney GM.** 2007. Semliki Forest virus nonstructural protein 2 is involved in suppression of the type I interferon response. J Virol **81:**8677–8684. http://dx.doi.org/10.1128/JVI.02411-06.
35. **Sumpter R, Jr, Loo YM, Foy E, Li K, Yoneyama M, Fujita T, Lemon SM, Gale M, Jr.** 2005. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. J Virol **79:**2689–2699. http://dx.doi.org/10.1128/JVI.79.5.2689-2699.2005.
36. **Bourai M, Lucas-Hourani M, Gad HH, Drosten C, Jacob Y, Tafforeau L, Cassonnet P, Jones LM, Judith D, Couderc T, Lecuit M, Andre P, Kummerer BM, Lotteau V, Despres P, Tangy F, Vidalain PO.** 2012. Mapping of Chikungunya virus interactions with host proteins identified nsP2 as a highly connected viral component. J Virol **86:**3121–3134. http://dx.doi.org/10.1128/JVI.06390-11.
37. **Treffers EE, Tas A, Scholte FE, Van MN, Heemskerk MT, de Ru AH, Snijder EJ, van Hemert MJ, van Veelen PA.** 2015. Temporal SILAC-based quantitative proteomics identifies host factors involved in chikungunya virus replication. Proteomics **15:**2267–2280. http://dx.doi.org/10.1002/pmic.201400581.
38. **Kam YW, Ong EK, Renia L, Tong JC, Ng LF.** 2009. Immuno-biology of Chikungunya and implications for disease intervention. Microbes Infect **11:**1186–1196. http://dx.doi.org/10.1016/j.micinf.2009.09.003.
39. **Sourisseau M, Schilte C, Casartelli N, Trouillet C, Guivel-Benhassine F, Rudnicka D, Sol-Foulon N, Le Roux K, Prevost MC, Fsihi H, Frenkiel MP, Blanchet F, Afonso PV, Ceccaldi PE, Ozden S, Gessain A, Schuffenecker I, Verhasselt B, Zamborlini A, Saib A, Rey FA, Arenzana-Seisdedos F, Despres P, Michault A, Albert ML, Schwartz O.** 2007. Characterization of reemerging chikungunya virus. PLoS Pathog **3:**e89. http://dx.doi.org/10.1371/journal.ppat.0030089.
40. **Mahata S, Bharti AC, Shukla S, Tyagi A, Husain SA, Das BC.** 2011. Berberine modulates AP-1 activity to suppress HPV transcription and downstream signaling to induce growth arrest and apoptosis in cervical cancer cells. Mol Cancer **10:**39. http://dx.doi.org/10.1186/1476-4598-10-39.
41. **Shin HB, Choi MS, Yi CM, Lee J, Kim NJ, Inn KS.** 2015. Inhibition of respiratory syncytial virus replication and virus-induced p38 kinase activity by berberine. Int Immunopharmacol **27:**65–68. http://dx.doi.org/10.1016/j.intimp.2015.04.045.
42. **Song S, Qiu M, Chu Y, Chen D, Wang X, Su A, Wu Z.** 2014. Down-regulation of cellular c-Jun N-terminal protein kinase and NF-kappaB activation by berberine may result in inhibition of herpes simplex virus replication. Antimicrob Agents Chemother **58:**5068–5078. http://dx.doi.org/10.1128/AAC.02427-14.
43. **Morton S, Davis RJ, McLaren A, Cohen P.** 2003. A reinvestigation of the multisite phosphorylation of the transcription factor c-Jun. EMBO J **22:**3876–3886. http://dx.doi.org/10.1093/emboj/cdg388.
44. **Thornton TM, Rincon M.** 2009. Non-classical p38 map kinase functions: cell cycle checkpoints and survival. Int J Biol Sci **5:**44–51.
45. **Vitari AC, Deak M, Collins BJ, Morrice N, Prescott AR, Phelan A, Humphreys S, Alessi DR.** 2004. WNK1, the kinase mutated in an inherited high-blood-pressure syndrome, is a novel PKB (protein kinase B)/Akt substrate. Biochem J **378:**257–268. http://dx.doi.org/10.1042/bj20031692.
46. **Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM.** 1998. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem **273:**18623–18632. http://dx.doi.org/10.1074/jbc.273.29.18623.
47. **Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC.** 1995. SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett **364:**229–233. http://dx.doi.org/10.1016/0014-5793(95)00357-F.
48. **Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW.** 2001. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci U S A **98:**13681–13686. http://dx.doi.org/10.1073/pnas.251194298.
49. **Tsetsarkin KA, Vanlandingham DL, McGee CE, Higgs S.** 2007. A single mutation in chikungunya virus affects vector specificity and epidemic potential. PLoS Pathog **3:**e201. http://dx.doi.org/10.1371/journal.ppat.0030201.
50. **Petersen LR, Powers AM.** 2016. Chikungunya: epidemiology. F1000Res **5:**pii:F1000 Faculty Rev-82. http://dx.doi.org/10.12688/f1000research.7171.1.
51. **Nunes MR, Faria NR, de Vasconcelos JM, Golding N, Kraemer MU, de Oliveira LF, Azevedo Rdo S, da Silva DE, da Silva EV, da Silva SP, Carvalho VL, Coelho GE, Cruz AC, Rodrigues SG, Vianez JL, Jr, Nunes BT, Cardoso JF, Tesh RB, Hay SI, Pybus OG, Vasconcelos PF.** 2015. Emergence and potential for spread of Chikungunya virus in Brazil. BMC Med **13:**102. http://dx.doi.org/10.1186/s12916-015-0348-x.
52. **Peng W, Peltier DC, Larsen MJ, Kirchhoff PD, Larsen SD, Neubig RR, Miller DJ.** 2009. Identification of thieno[3,2-b]pyrrole derivatives as novel small molecule inhibitors of neurotropic alphaviruses. J Infect Dis **199:**950–957. http://dx.doi.org/10.1086/597275.
53. **Zhao LJ, Wang W, Ren H, Qi ZT.** 2013. ERK signaling is triggered by hepatitis C virus E2 protein through DC-SIGN. Cell Stress Chaperones **18:**495–501. http://dx.doi.org/10.1007/s12192-013-0405-3.
54. **Smith JL, Stein DA, Shum D, Fischer MA, Radu C, Bhinder B, Djaballah H, Nelson JA, Fruh K, Hirsch AJ.** 2014. Inhibition of dengue virus replication by a class of small-molecule compounds that antagonize dopamine receptor d4 and downstream mitogen-activated protein kinase signaling. J Virol **88:**5533–5542. http://dx.doi.org/10.1128/JVI.00365-14.
55. **Voss K, Amaya M, Mueller C, Roberts B, Kehn-Hall K, Bailey C, Petricoin E, III, Narayanan A.** 2014. Inhibition of host extracellular signal-regulated kinase (ERK) activation decreases new world alphavirus multiplication in infected cells. Virology **468–470:**490–503. http://dx.doi.org/10.1016/j.virol.2014.09.005.

56. **Nakatsue T, Katoh I, Nakamura S, Takahashi Y, Ikawa Y, Yoshinaka Y.** 1998. Acute infection of Sindbis virus induces phosphorylation and intracellular translocation of small heat shock protein HSP27 and activation of p38 MAP kinase signaling pathway. Biochem Biophys Res Commun **253:**59–64. http://dx.doi.org/10.1006/bbrc.1998.9724.
57. **Su HL, Liao CL, Lin YL.** 2002. Japanese encephalitis virus infection initiates endoplasmic reticulum stress and an unfolded protein response. J Virol **76:**4162–4171. http://dx.doi.org/10.1128/JVI.76.9.4162-4171.2002.
58. **Huang M, Xu A, Wu X, Zhang Y, Guo Y, Guo F, Pan Z, Kong L.** 2016. Japanese encephalitis virus induces apoptosis by the IRE1/JNK pathway of ER stress response in BHK-21 cells. Arch Virol **161:**699–703. http://dx.doi.org/10.1007/s00705-015-2715-5.
59. **Fros JJ, Major LD, Scholte FE, Gardner J, van Hemert MJ, Suhrbier A, Pijlman GP.** 2015. Chikungunya virus non-structural protein 2-mediated host shut-off disables the unfolded protein response. J Gen Virol **96:**580–589. http://dx.doi.org/10.1099/vir.0.071845-0.
60. **Barry G, Fragkoudis R, Ferguson MC, Lulla A, Merits A, Kohl A, Fazakerley JK.** 2010. Semliki forest virus-induced endoplasmic reticulum stress accelerates apoptotic death of mammalian cells. J Virol **84:**7369–7377. http://dx.doi.org/10.1128/JVI.02310-09.
61. **Birkenkamp KU, Tuyt LM, Lummen C, Wierenga AT, Kruijer W, Vellenga E.** 2000. The p38 MAP kinase inhibitor SB203580 enhances nuclear factor-kappa B transcriptional activity by a non-specific effect upon the ERK pathway. Br J Pharmacol **131:**99–107. http://dx.doi.org/10.1038/sj.bjp.0703534.
62. **Henklova P, Vrzal R, Papouskova B, Bednar P, Jancova P, Anzenbacherova E, Ulrichova J, Maurel P, Pavek P, Dvorak Z.** 2008. SB203580, a pharmacological inhibitor of p38 MAP kinase transduction pathway activates ERK and JNK MAP kinases in primary cultures of human hepatocytes. Eur J Pharmacol **593:**16–23. http://dx.doi.org/10.1016/j.ejphar.2008.07.007.
63. **Aksamitiene E, Kiyatkin A, Kholodenko BN.** 2012. Cross-talk between mitogenic Ras/MAPK and survival PI3K/Akt pathways: a fine balance. Biochem Soc Trans **40:**139–146. http://dx.doi.org/10.1042/BST20110609.
64. **Kheir MM, Wang Y, Hua L, Hu J, Li L, Lei F, Du L.** 2010. Acute toxicity of berberine and its correlation with the blood concentration in mice. Food Chem Toxicol **48:**1105–1110. http://dx.doi.org/10.1016/j.fct.2010.01.033.
65. **Delang L, Segura Guerrero N, Tas A, Querat G, Pastorino B, Froeyen M, Dallmeier K, Jochmans D, Herdewijn P, Bello F, Snijder EJ, de Lamballerie X, Martina B, Neyts J, van Hemert MJ, Leyssen P.** 2014. Mutations in the chikungunya virus non-structural proteins cause resistance to favipiravir (T-705), a broad-spectrum antiviral. J Antimicrob Chemother **69:**2770–2784. http://dx.doi.org/10.1093/jac/dku209.
66. **Rulli NE, Rolph MS, Srikiatkhachorn A, Anantapreecha S, Guglielmotti A, Mahalingam S.** 2011. Protection from arthritis and myositis in a mouse model of acute chikungunya virus disease by bindarit, an inhibitor of monocyte chemotactic protein-1 synthesis. J Infect Dis **204:**1026–1030. http://dx.doi.org/10.1093/infdis/jir470.
67. **Liu SC, Lee HP, Hung CY, Tsai CH, Li TM, Tang CH.** 2015. Berberine attenuates CCN2-induced IL-1beta expression and prevents cartilage degradation in a rat model of osteoarthritis. Toxicol Appl Pharmacol **289:**20–29. http://dx.doi.org/10.1016/j.taap.2015.08.020.