Inhibition of the Extracellular Signal—Regulated Kinase/Ribosomal S6 Kinase Cascade Limits Chlamydia trachomatis Infection

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Chlamydia trachomatis is the cause of the most common bacterial sexually transmitted infection worldwide. Azithromycin is effective in treating chlamydial infection; however, resistance to this antibiotic is increasing, and it is important that new therapeutic strategies are developed. In this study, we demonstrated that inhibitors targeting each kinase in the extracellular signal—regulated kinase/ribosomal S6 kinase cascade significantly decreased the size and number of inclusions as well as the number of infectious progeny. The suppressive effects of the inhibitors were observed across the Chlamydia serotypes D, E, F, and L1 and across HeLa, McCoy, and Vero host cells. When combined with azithromycin, all the inhibitors exerted a synergistic suppressive effect on chlamydial infection. Knockdown experiments using small interfering RNA demonstrated that extracellular signal—regulated kinase 1/2 and ribosomal S6 kinase 1 were crucial for chlamydial infection. Moreover, BVD-523, a first-in-class extracellular signal—regulated kinase 1/2 inhibitor currently undergoing a phase II clinical trial, suppressed chlamydial infection both in cell culture and in a mouse model. These observations demonstrated not only that the extracellular signal—regulated kinase/ribosomal S6 kinase pathway plays a critical role in chlamydial infection but also that these kinases have potential as targets for host-directed therapy against C. trachomatis.


INTRODUCTION

Chlamydia trachomatis is the most common sexually transmitted bacterial pathogen worldwide (World Health Organization, 2016). It is estimated that there are approximately 100 million new cases of chlamydial infections annually (Newman et al., 2015). C. trachomatis serovars A–C cause infectious blindness, serovars D–K cause urinary or genital tract infections, and serovars L1–L3 are associated with lymphogranuloma venereum. Classically, lymphogranuloma venereum is characterized by the development of transient genital ulcer or papule, followed by the appearance of tender inguinal and/or femoral lymphadenopathy with a characteristic groove sign formed by swollen, matted lymph nodes developing along the course of the inguinal ligament.

Over the past decade, lymphogranuloma venereum has emerged in Europe and North America as a leading cause of proctitis and proctocolitis in men who have sex with men (Stoner and Cohen, 2015). Chlamydial infection can result in serious reproductive pathologies, including pelvic inflammatory disease, ectopic pregnancy, and infertility (Elwell et al., 2016; Menon et al., 2015). Azithromycin (AZM) is recommended as the first-line antimicrobial regimen for genital C. trachomatis infection according to the United States Centers for Disease Control and Prevention and the Chinese Center for Disease Control and Prevention (Qi et al., 2017; Workowski et al., 2015). However, a meta-analysis of C. trachomatis treatment reported that the cure rate for AZM was reduced from 97% to 94.4% from 2002 to 2014 (Kong et al., 2014; Lau and Qureshi, 2002). Moreover, a retrospective cohort study indicated that persistent and/or recurrent rectal chlamydial infection was found among 22% of male patients after treatment with AZM (Khosropour et al., 2014).

Host-directed therapy (HDT) is a novel approach for the treatment of bacterial and viral infections. HDT can improve host cellular responses to pathogens as well as target virulence factors and activate innate and adaptive immune responses (Zumla et al., 2016). Most HDT approaches are not stand-alone therapies but are combined with canonical anti-infectives (Kaufmann et al., 2018; Korbee et al., 2018). Currently, HDT is focused on Mycobacterium tuberculosis, hepatitis B virus, and HIV (Kaufmann et al., 2018; Rao et al., 2019; Verrier et al., 2017). Inosine-5’-monophosphate dehydrogenase was recently identified as a target for HDT,
efficiently inhibiting *C. trachomatis* growth both in vitro and in vivo (Rother et al., 2018). However, more potential targets remain to be discovered.

To treat *C. trachomatis* infection with HDT, the key host molecules that regulate bacterial infection should be identified as potential targets. *C. trachomatis*, an obligate intracellular bacterium, has two developmental forms: an infectious elementary body (EB) and a replicative reticulate body. Inside host cells, EBs reside within a vacuole (inclusion) and differentiate into reticulate bodies. Multiple host proteins are recruited to the inclusion and are functionally modulated to support chlamydial development. Several host signaling pathways play essential roles in chlamydial development, including the MAPK/extracellular signal–regulated kinase (ERK), Jak/signal transducer and activator of transcription, phosphoinositide 3-kinases/protein kinase B, and NF-κB pathways (Lad et al., 2005; Liu et al., 2019; Su et al., 2004). Ras/Raf/MAPK/ERK kinase (MEK)/ERK were reported to be activated during *C. trachomatis* infection (Du et al., 2011; Gurumurthy et al., 2010; Krüll et al., 2004). However, which ERK downstream molecules regulate *C. trachomatis* infection remains unknown. The main ERK substrates are ribosomal S6 kinase (RSK), MAPK-interacting kinase, mitogen- and stress-activated kinase, c-Fos and c-Jun, and calcium-dependent phospholipase A2 (cPLA2) (Slack et al., 2015). cPLA2 was initially reported to mediate chlamydial infection in human cell lines; however, this assertion was later challenged (Su et al., 2004; Vignola et al., 2010). The 90-kilodalton RSKs are a group of serine and/or threonine kinases comprising four RSK isoforms (RSK1–4) and are direct downstream effectors of ERK1/2 signaling (Houles and Roux, 2018; Samson et al., 2019). Although RSKs regulate numerous substrates involved in cell survival, growth, and proliferation (Houles and Roux, 2018; Samson et al., 2019), whether they have a role in chlamydial infection remains unknown.

In this study, we demonstrated that the activation of the ERK/RSK kinase cascade is essential for *C. trachomatis* infection. Small-molecule inhibitors of these kinases suppressed *C. trachomatis* infection in cell culture, whereas BVD-523 (ulixertinib) inhibited *C. trachomatis* infection in mice. We demonstrate the function of ERK/RSK signaling in chlamydial infection and present potential molecular targets for HDT to treat *C. trachomatis*.

**RESULTS**

**ERK1/2 is essential for *C. trachomatis* infection**

*C. trachomatis* infection is known to activate the MAPK/MEK/ERK, c-Jun N-terminal kinase, phosphoinositide 3-kinases/protein kinase B, and p38 signaling pathways (Capmany et al., 2019; Gurumurthy et al., 2010; Lad et al., 2005; Subbarayal et al., 2015). Therefore, we tested the effects of the small-molecule inhibitors LY294002 (targeting phosphoinositide 3-kinases), SP600125 (c-Jun N-terminal kinase pathway inhibitor), SB20390 (p38 MAPK inhibitor), AZD5363 (protein kinase B pathway inhibitor), and VX-11e (ERK inhibitor) on the growth of *C. trachomatis*. We found that VX-11e strongly inhibited chlamydial infection (Supplementary Figure S1). To further validate that ERK inhibition can suppress chlamydial infection, we tested another ERK inhibitor, BVD-523 (Mendzelevski et al., 2018; Smalley and Smalley, 2018). We concurrently compared the inhibitory effects of BVD-523 with those of U0126, a MEK inhibitor reported to suppress *C. trachomatis* infection (Gurumurthy et al., 2010; Krüll et al., 2004).

First, we performed a Cell Counting Kit-8 assay (Dojindo Laboratories, Beijing, China) to evaluate U0126, VX-11e, and BVD-523 toxicity. None of these inhibitors showed toxicity at concentrations below 20 μM (Supplementary Figure S2). Next, we performed a dose-dependent assay to determine the lowest dosages of the inhibitors that showed the strongest suppressive effect. *Chlamydia* infectivity and the number of infectious EB progeny decreased with increasing inhibitor concentrations (Supplementary Figure S3). On the basis of the assay results, 20 μM for U0126, 10 μM for VX-11e, and 20 μM for BVD-523 were used in subsequent experiments. The strongest depressive effect was observed at the earlier time points (0, 6, 12, 18, and 24 hours after infection) (Supplementary Figure S4). Finally, using the optimized dose and time point, we found that the inclusion bodies became smaller and the infectivity, inclusion size, and infectious progeny decreased (Figure 1a–d). Transmission electron microscopy (Japan Electron Optics Laboratory, Tokyo, Japan) revealed that substantially fewer EBs existed within shrunken inclusions (Figure 1e). VX-11e and BVD-523 exerted stronger inhibitory effects than U0126 (Figure 1c) (P < 0.001). These results demonstrated that VX-11e and BVD-523 inhibited chlamydial infection.

To confirm that ERK is a key effector for chlamydial infection, we knocked down ERK signaling with small interfering RNA (siRNA). Western blotting analysis confirmed the functionality of the ERK1- and ERK2-targeting siRNAs (Figure 1f). Inclusion number and size, infectivity, and infectious progeny production were decreased in host HeLa cells transfected with ERK1/2 siRNAs (Figure 1g–j). These results demonstrated that ERK1/2 is critical for chlamydial infection, and ERK1/2 inhibition suppresses *Chlamydia* replication.

**ERK1/2 inhibitors suppressed infection by different *Chlamydia* serotypes**

Because different *Chlamydia* serovars are known to cause different diseases, we investigated the effect of ERK1/2 inhibitors on serovars E, F, and L1. Similar to serovar D, inclusion number and size, infectivity, and the number of infectious serovar E, F, and L1 progeny were decreased after VX-11e and BVD-523 treatment (Figure 2). *C. trachomatis* serovars L1–L3 are associated with lymphogranuloma venereum, proctitis, and proctocolitis. Serovar L1 has enhanced virulence compared with that of serovar D, E, and F. It has the ability to infect cell monolayers effectively in the absence of centrifugation. Although serovar L1 has high infectivity in this experiment, it is still susceptible to ERK inhibitors. VX-11e and BVD-523 strongly suppress serovar L1 replication (Figure 2b and c) (P < 0.001). These results demonstrated that VX-11e and BVD-523 exerted inhibitory effects on *C. trachomatis* infection across different serovars.

**RSK1 is critical for chlamydial infection**

Although we found that ERK is critical for chlamydial infection, the downstream molecules involved in this effect
Figure 1. ERK1/2 is essential for Chlamydia trachomatis infection. (a) HeLa cells were infected with Chlamydia serovar D and exposed to 20 μM U0126, 10 μM VX-11e, or 20 μM BVD-523 for 48 hours. Chlamydia inclusions were stained with FITC-conjugated MOMP antibody (green), cytoplasm with Evan's blue (red), and nuclei with DAPI (blue) at ×400 magnification. Bar = 50 μm. (b) Infectivity, (c) inclusion size, (d) EB titer, and (e) TEM at ×6,000 magnification. (f) Western blotting was used to evaluate ERK1/2 protein expression in cells treated with siRNAs. (g) Microscopy assay, (h) infectivity, (i) inclusion size, and (j) EB titer were examined in infected HeLa cells transfected with ERK1/2-targeting siRNAs. Data represent the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001. (b, h) Fisher's exact test with Bonferroni's multiple comparisons, (c, i) Kruskal–Wallis followed by Dunn's multiple comparisons test, and (d, j) one-way ANOVA with Bonferroni's multiple comparisons test were used for statistical analysis. EB, elementary body; ERK, extracellular signal–regulated kinase; IFU, inclusion-forming unit; MOI, multiplicity of infection; ns, not significant; si ERK, small interfering ERK; siRNA, small interfering RNA; TEM, transmission electron microscopy.
remained unclear. RSK and cPLA2 are two key downstream molecules in the ERK pathway (Casalvieri et al., 2017; Su et al., 2004; Vignola et al., 2010). Therefore, we analyzed Chlamydia-infected cells exposed to MEK/ERK inhibitors at 48 hours after infection. Western blot analysis demonstrated that VX-11e and BVD-523 treatment reduced the levels of phosphorylated RSK (Figure 3a), which suggested that RSK might play an important role in Chlamydia replication. We tested this hypothesis using the RSK inhibitors LJH685 and LJI308. Smaller inclusions were observed by fluorescence microscopy (Olympus, Tokyo, Japan) and transmission electron microscopy (Figure 3b and c). Infectivity, inclusion size,
Figure 3. RSK1 is critical for Chlamydia trachomatis infection. HeLa cells were infected with Chlamydia and exposed to 80 μM LJH685 or 20 μM LJI308 for 48 hours. (a) Infected and uninfected HeLa cells exposed or not exposed to U0126, VX-11e, and BVD-523 by western blotting. (b) Representative images of chlamydial inclusions in infected HeLa cells exposed to 80 μM LJH685 or 20 μM LJI308. Bar = 50 μm. (c) Ultrastructural characteristics of chlamydial inclusions exposed to LJH685 or LJI308 at ×6,000 magnification. Bar = 2 μm. (d) Infectivity, (e) inclusion size, and (f) EB titer of Chlamydia were examined in cells exposed to LJH685 or LJI308. (g) RSK1 protein expression in cells transfected with siRNAs by western blotting. (h) Microscopy assay, (i) infectivity, (j) inclusion size, and (k) EB titer were examined in HeLa cells treated with RSK1-targeting siRNA. Means ± SDs of three independent experiments are shown. *P < 0.05, **P < 0.01, ***P < 0.001. (d, i) Fisher’s exact test with Bonferroni’s multiple comparisons, (e, j) Kruskal–Wallis followed by Dunn’s multiple comparisons test, and (f, k) one-way ANOVA with Bonferroni’s multiple comparisons were used for statistical analysis. cPLA2, calcium-dependent phospholipase A2; EB, elementary body; IFU, inclusion-forming unit; p-cPLA2, phosphorylated cPLA2; p-RSK, phosphorylated RSK; RSK, ribosomal S6 kinase; siRNA, small interfering RNA.
and infectious progeny decreased after LJH685 and LJI308 treatment (Figure 3d–f).

To determine the role of RSK in chlamydial infection development, we transfected HeLa cells with siRNA targeting RSK1 and found that RSK1 expression was reduced 48 hours after transfection (Figure 3g). We then infected HeLa cells with C. trachomatis serovar D and simultaneously transfected the cells with RSK1 siRNA. After 48 hours of culture, inclusions were smaller with RSK1 siRNA than with scramble control siRNA (Figure 3h). Infectivity, inclusion size, and infectious EB production were also decreased (Figure 3i–k). Together, these results suggested that RSK1 is critical for C. trachomatis infection.

ERK/RSK inhibitors and AZM synergistically suppress chlamydial infection

Because the ERK/RSK inhibitors impaired Chlamydia growth, we investigated whether ERK/RSK inhibitors and AZM would have a synergistic suppressive effect on chlamydial infection. HeLa cells were infected with Chlamydia and then exposed to U0126, VX-11e, BVD-523, LJH685, or LJI308 alone or with AZM for 48 hours. Inclusions become smaller, and their number was reduced with coexposure to AZM and ERK/RSK inhibitors (Figure 4a). The infectivity and the number of infectious progeny decreased with ERK/RSK inhibitors plus AZM treatment (Figure 4b and c). These results demonstrated that ERK/RSK inhibitors and AZM exerted synergistic repressive effects in chlamydial infection.

ERK/RSK inhibitors limited chlamydial infection in McCoy and Vero cells

We demonstrated that ERK/RSK inhibitors impaired chlamydial infection in HeLa cells, which are epithelial cells derived from human cervical cancer. Next, we examined whether the same inhibitory effect could be observed in different cell lines. McCoy cells are mouse fibroblasts, whereas Vero cells are African green monkey kidney cells, which are widely used cell models for chlamydial infection. As with HeLa cells, C. trachomatis serovar D was used to infect McCoy and Vero cells. Fluorescence microscopy analysis indicated that inclusion number and size in McCoy and Vero cells decreased with U0126, VX-11e, BVD-523, LJH685, and LJI308 treatment (Figure 5a). Infectivity and inclusion size decreased in both cell types after treatment with these inhibitors (Figure 5b and c). Moreover, the inhibitory effects of ERK/RSK inhibitors against chlamydial infection were time dependent in both McCoy and Vero cells (Supplementary Figure S5). These results indicated that ERK/RSK inhibitors could suppress infection by C. trachomatis in a wide range of host cells.

BVD-523 inhibited chlamydial infection in a mouse model

All the above experiments were performed with in vitro–cultured cells. Next, we tested the inhibitory effect of BVD-523 on chlamydial infection in a mouse model. We selected BVD-523 for the in vivo experiments because it is currently undergoing phase II clinical trials. We infected 6-week-old female BALB/c mice with Chlamydia serovar D to determine whether BVD-523 could inhibit Chlamydia development in vivo. DMSO, AZM, BVD-523, and AZM + BVD-523 were administered orally from day 5 to day 8 after infection. Vaginal swabs were taken on days 5 and 8 for cell culture and to determine the number of inclusions. No difference in the number of infectious progeny was recorded between day 5 and day 8 cultures (P > 0.05) (Figure 6a).

However, the number of progeny was greatly reduced in the AZM−, BVD-523−, and AZM + BVD-523−–treated groups on day 8 (P < 0.05) (Figure 6b–d). These data demonstrated that BVD-523 could inhibit C. trachomatis replication in vivo. There was no significant difference between AZM + BVD-523 combined treatment and either AZM or BVD-523 used alone by day 8 (P > 0.05).

Mice in the control group (infected with C. trachomatis but not treated) exhibited an enlarged uterus on day 7 after infection and polymuclear leukocytes (Supplementary Figure S6a). On day 21 after infection, H&E staining showed that fewer inflammatory cells were present in the uteruses of mice in the different treatment groups (Supplementary Figure S6b). H&E staining indicated that the heart, liver, spleen, and kidney of mice in the AZM and BVD-523 treatment groups were normal (Supplementary Figure S7), indicating that there were no systemic side effects.
Figure 4. ERK/RSK inhibitors and AZM synergistically suppressed chlamydial infection. (a) HeLa cells were infected with Chlamydia trachomatis serovar D and exposed to 0.02 μg/ml AZM, 20 μM U0126, 10 μM VX-11e, 20 μM BVD-523, 80 μM LJH685, and 20 μM LJI308 for 48 hours. Fluorescence images were captured at ×400 magnification. Bar = 50 μm. Shown are the (b) infectivity and (c) EB titer of Chlamydia in different treatments. Data represent mean ± SD from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001. (b) Fisher’s exact test with Bonferroni’s multiple comparisons and (c) one-way ANOVA with Bonferroni’s multiple comparisons test were used for statistical analysis. AZM, azithromycin; EB, elementary body; ERK, extracellular signal–regulated kinase; IFU, inclusion-forming unit; ns, not significant; RSK, ribosomal S6 kinase; siRSK, small interfering RSK.
drug target. Inosine-5’-monophosphate dehydrogenase, the rate-limiting enzyme in host-cell guanine nucleotide biosynthesis, efficiently inhibits Chlamydia growth both in vitro and in vivo (Rother et al., 2018). In this study, we showed that the ERK inhibitors BVD-523 and VX-11e effectively inhibited the in vitro growth of C. trachomatis across multiple cell lines. Moreover, BVD-523 inhibited Chlamydia replication in vivo. Although the most common treatment-related adverse events of BVD-523 were diarrhea, fatigue, nausea, and dermatitis acineform, BVD-523 has an acceptable safety profile with favorable pharmacokinetics (Sullivan et al., 2018). Small-molecule inhibitors of ERK signaling have potential as candidate drugs against C. trachomatis infection in HDT.

Figure 5. ERK/RSK inhibitors suppressed Chlamydia trachomatis infection in McCoy and Vero cells. (a) McCoy and Vero cells were infected with Chlamydia trachomatis serovar D and cultured in a medium containing 20 μM U0126, 10 μM VX-11e, 20 μM BVD-523, 80 μM LJH685, and 20 μM LJ308 for 48 hours. Fluorescence images were captured at ×400 magnification. Bar = 50 μm. Shown are the (b) infectivity and (c) inclusion size of C. trachomatis in cells treated with MEK/ERK/RSK inhibitors. Data represent mean ± SD from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001. (b) Fisher’s exact test with Bonferroni’s multiple comparisons and (c) Kruskal–Wallis followed by Dunn’s multiple comparisons were used for statistical analysis. ERK, extracellular signal–regulated kinase; MEK, MAPK/ERK kinase; ns, not significant; RSK, ribosomal S6 kinase.

Figure 6. BVD-523 inhibited chlamydial infection in a mouse model. Two mg/kg AZM was provided by gastrointestinal administration at the beginning of day 5. Sixty mg/kg BVD-523 was provided by gastrointestinal administration from day 5 to day 8. For AZM+BVD-523 treatment group, mice were treated with 2 mg/kg AZM and 60 mg/kg BVD-523 from day 5 to day 8. Vaginal swabs were taken on days 5 and 8 for cell culture and determination of the number of inclusions. (a) Control group. There was no significant difference in inclusion formation between days 5 and 8 (ns, P > 0.05). (b) AZM treatment group. After 4 days of treatment, the number of infectious Chlamydia was significantly reduced (*P < 0.05). (c) BVD-523 treatment group (*P < 0.05). (d) AZM + BVD-523 treatment group (*P < 0.05). Nonparametric Wilcoxon test was used for statistical analysis. AZM, azithromycin; IFU, inclusion-forming unit; ns, not significant.
RSK is an important downstream transducer in the ERK signaling pathway, and ERK inhibition has been shown to decrease RSK phosphorylation (Roskoski, 2019). Recent clinical trials for BVD-523 have selected phosphorylated RSK as a pharmacodynamic marker to evaluate the response to ERK inhibitors (NCT02296242) (Germann et al., 2017). Similarly, ERK inhibition by VX-11e treatment resulted in robust suppression of phosphorylated RSK and consequent tumor growth inhibition (Krepler et al., 2016). In this study, we demonstrated that the RSK inhibitors LJH685 and LJ308 reduced chlamydial infection in three cell lines, whereas siRNA-mediated RSK knockdown inhibited chlamydial infection, indicating that RSK may play an important role in Chlamydia replication. This study demonstrated that the ERK/RSK axis is critical for chlamydial infection.

Most HDT approaches are combined with canonical anti-infectives. In this study, we demonstrated that the ERK/RSK inhibitors VX-11e, BVD-523, LJH685, and LJ308 exert synergistic effects with AZM in chlamydial infection. ERK and RSK are potential drug targets for Chlamydia HDT. The clinical evaluation of the HDT for chlamydial infection requires further investigation.

In summary, we have provided evidence that the ERK/RSK cascade plays a critical role in C. trachomatis infection. We have also demonstrated that ERK/RSK inhibitors reduced Chlamydia replication in vitro, whereas the ERK1/2 inhibitor, BVD-523, inhibited chlamydial infection in vivo. This indicates that the factors involved in the ERK/RSK cascade may be targets for drug development in the treatment of C. trachomatis.

MATERIAL AND METHODS
Cell culture and chlamydial infection
Human epithelial carcinoma cells (HeLa) (ATCC, Manassas, VA; CCL-2.1), mouse fibroblast cells (McCoy) (kindly provided by Lilang Jiang, Sun Yat-sen University, China), and African green monkey kidney cells (Vero) (CCTCC, Wuhan, China; GDC062) were cultured in DMEM supplemented with 10% fetal calf serum. Cells were grown at 37 °C and 5% carbon dioxide. C. trachomatis serovars D, E, F, and L1 were provided by Joke Spaargaren of the Public Health Laboratory of the Municipal Health Service of Amsterdam, Netherlands. HeLa, McCoy, Vero, and C. trachomatis strains were negative for mycoplasma infection as determined by a Public Health Laboratory of the Municipal Health Service of Amsterdam, Netherlands. HeLa, McCoy, Vero, and C. trachomatis strains were negative for mycoplasma infection as determined by the manufacturer’s instructions (Biological Industries, Tel Aviv, Israel). Chlamydia was propagated as previously described (Xue et al., 2017).

Inhibitors, antibodies, and antibiotic
Inhibitors of LY294002, SB20190, MEK1/2 UO126, VX-11e, BVD-523, LJH685, LJ308, SP600125, and AZD5363 were bought from MedChemExpress (Monmouth Junction, NJ). Antibodies against GAPDH, phosphorylated ERK, phosphorylated RSK, phosphorylated cPLA2, total ERK, total RSK, and total cPLA2 were from Cell Signaling Technology (Danvers, MA). The inclusions were observed by the FITC-conjugated antibody against the MOMP of C. trachomatis (Trinity Biotech, Wicklow, Ireland). AZM (North China Pharmaceutical Group Corporation, Hebei, China) was dissolved in ethanol.

Cell viability assay
Cells were seeded in 96-well plates at a density of 5 × 10^4 cells per well. The effects of DMSO and signaling pathway inhibitors on HeLa, McCoy, or Vero cells were determined using a Cell Counting Kit-8 assay.

Immunofluorescence staining
Infected cells were cultured on glass coverslips and fixed in 4% paraformaldehyde at 48 hours after infection. The cytoplasm was stained with Evans blue (Macklin, Shanghai, China), and nuclei were counterstained with DAPI. Chlamydia inclusion was stained with FITC-conjugated MOMP by fluorescence microscopy at ×400 magnification.

Confocal microscopy analysis
Confocal images of chlamydial inclusions and nuclei were captured using Nikon A1R software (Nikon, Tokyo, Japan) at ×200 magnification. Nikon A1R NIS-Elements C software was used to analyze the images. All images used in intensity measurements were obtained under standard exposure.

Transmission electron microscopy
Cells were collected and pelleted by centrifugation at 1,000g for 5 minutes and stored at 4 °C. Thin sections were examined by transmission electron microscopy operating at 100 kV.

Infectivity assay
Infectivity was calculated as the inclusion and/or nuclei percentage. In each experiment, the number of inclusions and nuclei were counted in 15 random fields from triplicate samples by confocal microscopy at ×200 magnification. The average percentage was calculated using Nikon A1R NIS-Elements C software.

Inclusion size measurement
Five images of random fields were taken for each sample at ×200 magnification using confocal microscopy. The area of 50–60 inclusions per sample was measured for each experimental condition.

EB titer analysis
EBs were released from the cells by sonication and used to infect fresh monolayer. Total inclusions were counted at ×200 magnification. The average number of inclusion-forming units/ml was calculated per sample and experimental condition.

Western blot analysis
Cells were lysed in 1 × SDS-PAGE loading buffer containing 50 mM β-glycerophosphate (Merck, Kenilworth, NJ). The lysates were transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) for western blot analysis.

siRNA transfection
siRNA sequences were designed to target ERK1 mRNA, ERK2 mRNA (Gunawardhana et al., 2018; Kolb et al., 2012), or RSK1 mRNA (Supplementary Table S1). A nontargeting siRNA was used as a negative control for all the siRNA transfection experiments. All the siRNAs were synthesized by Ribobio (Guangzhou, China). siRNA transfections were performed according to the manufacturer’s instructions. Briefly, HeLa cells were infected with Chlamydia and transfected with the appropriate siRNA with RNAiMAX (Invitrogen, Carlsbad, CA) for 48 hours.

Animals
Female BALB/c mice (4–6 weeks old) were purchased from the Animal Core Facility of Southern Medical University (Guangzhou, China). The mice were housed in a clean environment at 24 ± 2 °C.
and an alternating 12-hour light and/or dark cycle for 1 week. They were then subcutaneously injected with 2.5 mg of Depo-Provera (Baiyunshan Pharmaceutical Company, Guangzhou, China) to synchronize their menstrual cycles and increase infection efficiency (Phillips Campbell et al., 2012). After 1 week of treatment, mice were vaginally infected with $1 \times 10^6$ inclusion-forming units of C. trachomatis or an equal volume of 2SPG. Mice were killed at 21 days after infection. The study was approved by the Animal Care and Use Committee of Dermatology Hospital of Southern Medical University.

**Drug treatment in vivo**

BVD-523 was dissolved in DMSO at 100 mg/ml, and AZM was dissolved in ethanol at 5 mg/ml. Both were diluted in 5% glucose. Mice were randomly divided into four groups administered DMSO, AZM, BVD-523, or AZM + BVD-523 by gastrointestinal administration. Mice were treated with 2 mg/kg AZM once or 60 mg/kg BVD-523 twice daily by gastrointestinal gavage for 4 days beginning on day 5 after infection (Germann et al., 2017). Vaginal swabs were taken on days 5 and 8 for cell culture and to determine the number of inclusions.

**Statistical analysis**

GraphPad Prism 5 (GraphPad Software, La Jolla, CA) was used for graphing, and data analysis was performed using SPSS 15.0 (SPSS, Chicago, IL). Quantitative data were presented as mean ± SD. The quantitative data were tested for normality using the Shapiro–Wilks test. Fisher’s exact test with Bonferroni’s multiple comparisons was used when assessing infectivity. Kruskal–Wallis followed by Dunn’s multiple comparisons test was used for evaluating the inclusion area. One-way ANOVA with Bonferroni’s multiple comparisons test was used for evaluating EB titer. Nonparametric Wilcoxon test was used for mouse model statistics. Differences were considered significant at *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

**DATA AVAILABILITY STATEMENT**

No database was involved in this study.

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**CONFLICT OF INTEREST**

The authors state no conflicts of interest.

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**AUTHOR CONTRIBUTIONS**

Conceptualization: HZ, YX, ZR, WC, CW; Formal Analysis: WC; Funding Acquisition: YX, HZ; Investigation: YX, WC, ZM, XY, QW, YW; Methodology: HZ, ZR, YX; Project Administration: ZR, HZ; Supervision: ZR, HZ; Visualization: YX, WC; Writing - Original Draft Preparation: YX; Writing - Review and Editing: ZR, HZ

**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.07.033.

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ERK/RSK Inhibitors Limit Chlamydia Infection

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Supplementary Figure S1. Inhibitors targeting PI3K/Akt and MAPK kinases show different repressive activities against Chlamydia trachomatis infection. We tested the effects of the small molecule inhibitors LY294002 (targeting PI3 kinase), SP600125 (JNK pathway inhibitor), SB20190 (p38 MAPK inhibitor), AZD5363 (Akt pathway inhibitor), and VX-11e (ERK inhibitor) on the growth of C. trachomatis. 10 μM LY294002, 10 μM SB20190, 10 μM VX-11e, 10 μM SP600125, and 10 μM AZD5363 were used. (a) LY294002, SP600125, SB20190, AZD5363, and VX-11e are inhibitors of PI3K, JNK, p38-MAPK, Akt, and ERK, respectively. Cells were exposed to the inhibitors and simultaneously infected with Chlamydia. (b) The inhibitors were added to the medium at 24 hours after infection. Bar = 100 μm. Akt, protein kinase B; ERK, extracellular signal–regulated kinase; JNK, c-Jun N-terminal kinase; PI3K, phosphoinositide 3-kinase.

Supplementary Figure S2. Evaluation of cell toxicity of the MEK/ERK inhibitors. HeLa cells were treated with DMSO, U0126, VX-11e, or BVD-523 at various concentrations for 48 hours, followed by the CCK-8 assay. HeLa cells were exposed to 2.5 μM U0126, 5.0 μM U0126, 10.0 μM U0126, 20.0 μM U0126, 40.0 μM U0126, 80.0 μM U0126, 2.5 μM VX-11e, 5.0 μM VX-11e, 10.0 μM VX-11e, 20.0 μM VX-11e, 40.0 μM VX-11e, 80.0 μM VX-11e, 2.5 μM BVD-523, 5.0 μM BVD-523, 10.0 μM BVD-523, 20.0 μM BVD-523, 40.0 μM BVD-523, 80.0 μM BVD-523 for 48 hours. *P < 0.05, **P < 0.01, ***P < 0.001. Fisher’s exact test with Bonferroni’s multiple comparisons was used for statistical analysis. CCK-8, cell counting kit-8; ERK, extracellular signal–regulated kinase; MEK, MAPK/ERK kinase; ns, not significant.
Supplementary Figure S3. The inhibitory effects of VX-11e and BVD-523 on *Chlamydia trachomatis* infection were dose dependent. HeLa cells were exposed to 5.0 μM U0126, 10.0 μM U0126, 20.0 μM U0126, 40.0 μM U0126, 80.0 μM U0126, 5.0 μM VX-11e, 10.0 μM VX-11e, 20.0 μM VX-11e, 40.0 μM VX-11e, 80.0 μM VX-11e, 5.0 μM BVD-523, 10.0 μM BVD-523, 20.0 μM BVD-523, 40.0 μM BVD-523, or 80.0 μM BVD-523 for 48 hours. (a) Infectivity and (b) EB titer of *C. trachomatis* were examined in cells treated with various concentrations of U0126, VX-11e, or BVD-523. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the DMSO control. (a) Fisher's exact test with Bonferroni's multiple comparisons and (b) one-way ANOVA with Bonferroni's multiple comparisons test were used for statistical analysis. EB, elementary body; IFU, inclusion-forming unit.
Supplementary Figure S4. The inhibitory effects of VX-11e and BVD-523 on Chlamydia trachomatis infection were time dependent. HeLa cells were infected with Chlamydia and exposed to 20 μM U0126, 10 μM VX-11e, or 20 μM BVD-523 for 48 h. (a) C. trachomatis inclusions were stained with FITC-conjugated MOMP antibody. (b) Infectivity and (c) EB titer were analyzed. Bar = 50 μm. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the DMSO control. (b) Fisher’s exact test with Bonferroni’s multiple comparisons and (c) one-way ANOVA with Bonferroni’s multiple comparisons test were used for statistical analysis. EB, elementary body; h, hour; IFU, inclusion-forming unit.
Supplementary Figure S5. The inhibitory effects of VX-11e and BVD-523 on *Chlamydia trachomatis* infection were time dependent in McCoy and Vero cells. McCoy cells were infected with *Chlamydia* and exposed to 10 μM VX-11e, or 20 μM BVD-523 for 48 hours. (a) Infectivity and (b) EB titer were analyzed. (c, d) Similar experiments as in a and b except that experiments were performed with Vero cells instead of McCoy cells. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the DMSO control. (a) Fisher's exact test with Bonferroni's multiple comparisons and (b) one-way ANOVA with Bonferroni's multiple comparisons tests were used for statistical analysis. EB, elementary body; h, hour; IFU, inclusion-forming unit; ns, not significant.
Supplementary Figure S6.
Pathological changes in the uteruses of mice treated with BVD-523. 2 mg/kg AZM and 60 mg/kg BVD-523 was provided by gastrointestinal administration. For AZM + BVD-523 treatment group, mice were treated with 2 mg/kg AZM and 60 mg/kg BVD-523 from day 5 to day 8. (a) Macroscopy and H&E staining of normal and infected mouse uteruses on day 7 after infection with *Chlamydia trachomatis* serovar D. (b) Macroscopy and H&E staining of mouse uteruses on day 21 after infection with *C. trachomatis* serovar D and treated as indicated. Bars = 200 μm (for middle images in panels a and b) and = 50 μm (for images on the right side in panels a and b). AZM, azithromycin.
Supplementary Figure S7. The toxicity of BVD-523 in vivo. The hearts, livers, spleens, and kidneys were harvested and stained with H&E 16 days after treatment with AZM, BVD-523, and AZM + BVD523. 2 mg/kg AZM and 6 mg/kg BVD-523 was provided by gastrointestinal administration. For AZM + BVD-523 treatment group, mice were treated with 2 mg/kg AZM and 60 mg/kg BVD-523 from day 5 to day 8. Bar = 50 μm. AZM, azithromycin.