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Inhibition of polyamine biosynthesis is a broad spectrum antiviral strategy against RNA viruses.

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Emerging viruses present an extraordinary threat to human health, given their sudden and unpredictable appearance and the potential for rapid spread among the human population. Recent emergence of chikungunya virus (CHIKV) in the Americas¹, Middle East respiratory syndrome coronavirus (MCoV) in the Arabian peninsula² and Ebola virus in Western Africa³ highlight the struggles to contain outbreaks. A significant hurdle is the availability and implementation of antiviral therapies to treat the infected or protect at-risk populations, such as family members and healthcare workers. While several compounds show promise *in vitro* and *in vivo*, these recent outbreaks underscore the need to accelerate drug discovery, as well as to explore therapeutic avenues of broad antiviral activity. In this report, we describe the antiviral effects of difluoromethylornithine (DFMO, eflornithine), a potent suicide inhibitor of ornithine decarboxylase (ODC1)⁴, a critical enzyme in polyamine synthesis. We show that DFMO is active against diverse families of RNA viruses. Our data show that polyamines are a general requirement for viral RNA synthesis. DFMO is bioavailable and currently used in treating trypanosomiasis⁵ and hirsutism⁶, and given its tolerance in humans⁷, may be an immediately available and viable option for controlling infection during outbreaks of significant concern.

Polyamines are small, positively-charged molecules involved in several cellular processes, including proliferation⁸, apoptosis⁹, ion channel regulation¹⁰, DNA conformation¹¹, and transcription^{12,13}. The biosynthesis of polyamines is regulated by several enzymes, but the conversion of ornithine into putrescine by ornithine decarboxylase (ODC1) is the bottleneck step¹⁴. Although early work shows that polyamines are included in virions¹⁵⁻¹⁷ or facilitate viral replication^{18,19}, their general role in viral infection is not established for RNA viruses^{20,21}. We utilized CHIKV to study the role of polyamines in alphavirus infection. CHIKV titers were significantly

reduced ($p=0.010$) in BHK-21 cells treated for four days with 500 μM DFMO and completely rescued with the addition of exogenous polyamines (**Fig. 1a**). The complete phenotypic reversal with exogenous polyamines suggests that DFMO treatment was non-toxic to cells, which we corroborated with viability assays (**Supplementary Fig. 1**). The biogenic polyamines putrescine (Put) and spermidine (Spd) also fully rescued viral titers when added individually, but the polyamine precursor, ornithine (Orn), did not (**Supplementary Fig. 2**), indicating that the biogenic polyamines are required for robust viral replication. Additionally, CHIKV titers were not enhanced with polyamine treatment alone, indicating that viral replication is maximized with endogenous levels of polyamines (**Supplementary Fig. 3**). We observed the same phenotype in *Aedes albopictus* C6/36 cells (**Fig. 1b**), primary C57/Bl6 murine embryonic fibroblasts (**Fig. 1c**), and green monkey kidney Vero-E6 cells (**Fig. 1d**). For Sindbis virus (SINV), a closely-related alphavirus, we also observed reduced titers ($p=0.002$) with DFMO treatment and full rescue with exogenous polyamines (**Fig. 1e**).

To confirm that DFMO was reducing polyamine content of treated cells, we performed immunofluorescence with an antibody directed against the biogenic polyamines (spermine, spermidine, and putrescine). BHK-21 cells were treated with DFMO and subsequently infected with CHIKV containing mCherry-tagged nsp3²² to visualize replication compartments. Upon DFMO treatment, polyamine signal was significantly reduced (**Fig. 1f**). With CHIKV infection, no gross difference was noted in polyamine content or localization, and polyamines did not appear to localize precisely with replication compartments. In addition, we directly visualized individual polyamines within untreated or DFMO-treated cells by thin-layer chromatography²³. DFMO treatment reduced the total amount of polyamines by approximately 73% (**Fig 1g**). As a further confirmation that polyamines are critical to CHIKV replication, we used another ODC1 inhibitor, POB, and observed similar reduction in viral titers (**Fig. 1h**).

DFMO-mediated knockdown of viral titers of Semliki Forest Virus relied on a four-day pretreatment²⁴. For CHIKV, BHK-21 cells required a 48-hour pretreatment to significantly impact titers ($p=0.038$), further reduced with 72- or 96-hour pretreatment (**Fig. 1i**). This pretreatment was effective at MOI 0.1 and 1, but not 10 for both CHIKV and SINV (**Supplementary Fig. 4**).

Previous reports described a role for polyamines in herpes simplex virus-1²⁵, Semliki Forest virus²⁴, and hepatitis C virus²⁶ polymerase activity or RNA replication, and human cytomegalovirus viral assembly²⁷. Entry of CHIKV was not impacted, as viral RNA was equivalent ($p=0.282$) in untreated and DFMO-treated cells at 2 hours post infection (**Fig. 2a**). Furthermore, we observed significant inhibition of RNA synthesis for both genomic ($p=0.001$) and subgenomic ($p=0.039$) messengers at 24 hours (**Fig. 2a, b**). SINV viral genomes were similarly reduced (**Fig. 2c**). Although polyamines did not specifically localize with replication compartments as assayed by immunofluorescence (**Fig. 1f**), we asked whether polyamines interact with the replication complex or with viral RNA. BHK-21 cells were infected for 16 hours, lysed and immunoprecipitated with an anti-polyamine antibody to analyze co-immunoprecipitation of replication components. The viral nsp2 (helicase, methyltransferase), nsp3 and nsp4 (polymerase) were indeed found to co-immunoprecipitate (**Fig. 2d**). To provide additional evidence of this interaction, we performed RNA immunoprecipitation with the same anti-polyamine antibody. Both genomic and subgenomic RNA co-immunoprecipitated with polyamines (**Fig. 2e**), further implicating them in viral RNA replication.

To test whether polyamines directly stimulate viral transcription, we measured polymerase activity of both CHIKV and SINV using an *in vitro* replication (IVR) assay with replication complexes purified from infected cells. Membrane fractions from infected cells were incubated in buffer alone, or buffer containing exogenous polyamines, and polymerization was measured by radionucleotide incorporation after a 3-hour incubation. Samples incubated with polyamines exhibited a significant

increase ($p=0.021$ and $p=0.015$ for CHIKV and SINV, respectively) in viral RNA synthesis (**Fig. 2f**, quantitated in **Fig. 2g, h**). Similar analyses by qRT-PCR and using the CHIKV replicon system exhibited the same phenotype (**Fig. 2i**). In these assays, polyamines could be either directly stimulating the replication complex, or stabilizing templates and nascent genomes. To discern between these hypotheses, viral RNA was incubated in IVR reaction mixtures using membranes from uninfected cells, in the presence or absence of polyamines. Rather than stabilizing viral RNA, we observed significant degradation of viral genomes when incubated with polyamines (**Supplementary Fig. 5**). Taken together, our data support that polyamines are directly stimulating replication complexes activity.

To understand the sensitivity of CHIKV to DFMO, we infected BHK-21 cells treated with a range of concentrations, from 1 μM to 10 mM and determined the IC_{50} to be 35.55 μM (13.05 to 96.08 μM) (**Fig. 3a**), which is near the measured plasma concentration in patients treated with oral DFMO⁷. At concentrations above 100 μM , titers were significantly reduced ($p<0.002$ for each), and no virus was recovered with 10 mM treatment. Similar results were obtained for SINV (**Supplementary Fig. 6**). Additionally, a 2013 Caribbean strain of CHIKV exhibited significant sensitivity to DFMO that was completely reversed with exogenous polyamine treatment (**Fig. 3b**).

While the required pre-exposure treatment may be a viable option for susceptible populations during an outbreak, such as medical professionals treating infected individuals or cohabitants of individuals with communicable diseases, treating the broader population prior to infection is impractical for alphaviruses. To test whether DFMO could reduce viral titers if given after infection, we infected BHK-21 cells with CHIKV at a MOI of 0.0001 (100 viral particles), to mimic a more natural infectious dose, and treated cells with 1 mM DFMO just after infection. CHIKV titers were significantly reduced ($p=0.0004$) by 32 hours and remained significantly suppressed ($p<0.003$) through 72 hours (**Fig. 3c**). To further investigate potential clinical applications of DFMO, we examined whether it could be combined with

another antiviral pharmaceutical, ribavirin²⁸. We found that DFMO synergizes with 400 μ M ribavirin to significantly reduce CHIKV titers ($p=0.038$ and 0.0553) in BHK-21 cells beyond either of the antivirals individually (**Fig. 3d**).

Polyamines are ubiquitous molecules and as such, we wished to determine whether their requirement, and the effects of DFMO that we observed for alphaviruses, were more broadly applicable. Thus, we explored several RNA viruses from different taxonomic families: the positive-sense coronavirus Middle Eastern respiratory syndrome virus (MCoV, **Fig. 3e**); the positive-sense picornaviruses coxsackievirus B3 (CVB3, **Fig. 3f**), poliovirus (PV, **Fig. 3g**), and enterovirus-71 (EV-71, **Fig. 3h**); the positive-sense flaviviruses dengue fever virus-1 (DENV1, **Fig. 3i**), Zika virus (ZIKV, **Fig. 3j**), Japanese encephalitis virus (JEV, **Fig. 3k**), yellow fever virus (YFV, **Fig. 3l**), and West Nile virus (WNV, **Fig. 3m**); the negative-sense rhabdoviruses vesicular stomatitis virus (VSV, **Fig. 3n**) and rabies virus (RABV, **Fig. 3o**); and the negative-sense, segmented bunyavirus Rift Valley fever virus (RVFV, **Fig. 3p**). Despite the diversity in viral family and cell types, DFMO treatment significantly reduced viral titers for all viruses ($p<0.05$ for all), and in all cases the infection phenotype was rescued by the addition of exogenous polyamines. These results reveal that polyamines may be a general requirement for the replication of all RNA viruses.

Our results uncover a universal role of polyamines in RNA virus replication and a broad antiviral activity of DFMO against every RNA virus we tested. Although the relatively long pretreatment time required to knockdown endogenous polyamines precludes the use of DFMO in most situations of acute infection, we propose DFMO as a potential preventative or early-response therapeutic in particularly severe outbreaks. Given that DFMO is already clinically approved for treating African sleeping sickness in humans^{5,29}, the rapid implementation of DFMO during serious outbreaks may warrant immediate attention. Specifically, this relatively well-tolerated drug could provide added protection for at-risk populations, such as healthcare

workers or family members of afflicted individuals, to prevent spread of disease. Furthermore, since many RNA virus infections are characterized by several days of incubation before onset of symptoms, treatment of individuals soon after suspected exposure may delay replication or reduce titers enough, in favor of the mounting immune response, to reduce mortality. Derivatives of DFMO and other molecules that manipulate the polyamine biosynthetic pathway have been developed as anti-cancer agents³⁰ as well as for the treatment of leishmaniasis³¹. These drugs should also be examined as potent inhibitors of RNA viruses.

Acknowledgments

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Author Contributions

B.C.M., E.Z.P. G.M., E.C.S., G.B-S., C.D., C.K., M-P.F., R.V., S.V. E.S-L., M.P., and K.A.S. performed experiments. Experiments were designed by A.S., E.S-L. and M.P. for DENV1 and ZIKV, M.L. and S.V. for RABV, M.R.D. and E.C.S. for MCoV, N.P., C.K. and M-P.F. for JEV and WNV, G.B-S. for YFV, F.D. and R.V. for PV and EV-A71, M.F. and C.D. for RVFV, and M.V. for CHIKV, SINV, CVB3, and VSV. B.C.M., E.Z.P., and M.V. designed additional experiments and analyzed data. B.C.M. and M.V. wrote the manuscript.

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Figure Legends

Figure 1. DFMO exhibits antiviral activity against alphaviruses. Chikungunya virus (CHIKV) titers in (a) baby hamster kidney (BHK) fibroblasts, (b) *Aedes albopictus* (C6/36) cells, (c) C57/BI6 murine embryonic fibroblasts, or (d) Vero-E6 epithelial cells treated for four days with 500 μ M DFMO or in combination with exogenous polyamines and infected for 24 h. (e) Sindbis virus (SINV) titers in BHK fibroblasts treated for four days with 500 μ M DFMO. (f) Representative immunofluorescence against polyamines (green) and mCherry-tagged viral nsp3 in untreated and DFMO-treated (500 μ M, four days) mock- or CHIKV-infected BHK cells for 8 h. (g) Representative thin layer chromatography analysis of cellular lysates from BHK cells treated with 500 μ M DFMO or in combination with exogenous polyamines. Individual polyamines are labeled for putrescine (put), spermidine (spd), and spermine (spm). (h) CHIKV titers in BHK cells treated with the ODC1 inhibitor, POB (200 μ M, four day pretreatment, 24 hpi). (i) CHIKV titers from DFMO-treated BHKs with increasing pretreatment times. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$ versus untreated control or as indicated, one-tailed Student's T-test, n=3. Error bars represent mean \pm one standard deviation. Immunofluorescence images and chromatograph are representative of three and two independent replicates, respectively.

Figure 2. Polyamines stimulate RNA-dependent RNA polymerase activity. (a) Time-course of CHIKV genomes upon four-day 500 μ M DFMO treatment (n=3). (b) CHIKV subgenomes and (c) SINV genomes as quantitated by qRT-PCR, following four-day 500 μ M DFMO treatment and 24-h infection (n=4). (d) Western blot of coimmunoprecipitation of nsp2, nsp3, and nsp4 with polyamines (representative of two independent replicates). (e) RNA immunoprecipitation of CHIKV genomes and

subgenomes with nsp2 and polyamine antibodies (n=4). **(f)** Agarose gel analysis of *in vitro* replicated SINV and CHIKV genomes, as indicated, with or without supplementation with polyamines. Quantitation of SINV **(g)** and CHIKV **(h)** bands from **f** (n=2). **(i)** qRT-PCR analysis of genome replication following four-day 500 μ M DFMO treatment and transfection with viral replicon (n=3). Error bars represent mean \pm one standard deviation. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$ comparing DFMO treatment to untreated control or as indicated, one-tailed Student's T-test, n-values are indicated. IVR image is representative of three independent replicates.

Figure 3. DFMO is broadly antiviral. **(a)** CHIKV titers at 24 hpi following four-day pretreatment with increasing concentrations of DFMO in BHK cells (n=3). **(b)** Caribbean-isolated CHIKV titers in BHK cells following DFMO pretreatment and 24-h infection (n=3). **(c)** CHIKV titer time-course following inoculation at MOI 0.0001 (10 PFU) and DFMO treatment 1 hpi at 1 mM (n=3). **(d)** Effect of DFMO pretreatment, ribavirin treatment, and combined treatment, on CHIKV titers (n=2). **(e-p)** Virus titers following DFMO treatment and rescue with exogenous polyamines. **(e)** Middle East respiratory syndrome coronavirus (MCoV) at 24 hpi in Vero81 cells **(f)** Coxsackvirus B3 (CVB3) titers at 24 hpi in HeLa cells **(g)** Poliovirus (PV) at 24 hpi in HeLa cells **(h)** Enterovirus-A71 (EV-A71) after 24-h infection of HeLa cells **(i)** Dengue virus-1 (DENV1) at 24 hpi in BHK-21 cells **(j)** Zikavirus (ZIKV) at 24 hpi in BHK-21 cells **(k)** Japanese encephalitis virus (JEV) at 24 hpi in BHK-21 cells **(l)** Yellow fever virus (YFV) at 96 hpi in BHK-21 **(m)** West Nile virus (WNV) at 24 hpi in BHK-21 cells **(n)** Vesicular stomatitis virus (VSV) at 16 hpi in BHK-21 cells at MOI 0.1. **(o)** Rabies virus (RABV) somatic inclusions measured in fluorescent intensity per cell at 24 hpi in primary cortical neurons **(p)** Rift Valley fever virus (RVFV) at 24 hpi in BHK-21 cells. * $P \leq 0.05$ versus untreated control. Error bars represent mean \pm one standard deviation. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$ versus untreated control or as

indicated, one-tailed Student's T-test, n=3 replicates for all viruses except DENV (n=8), ZIKV (n=9), MERS (n=6), and RABV (n=6).

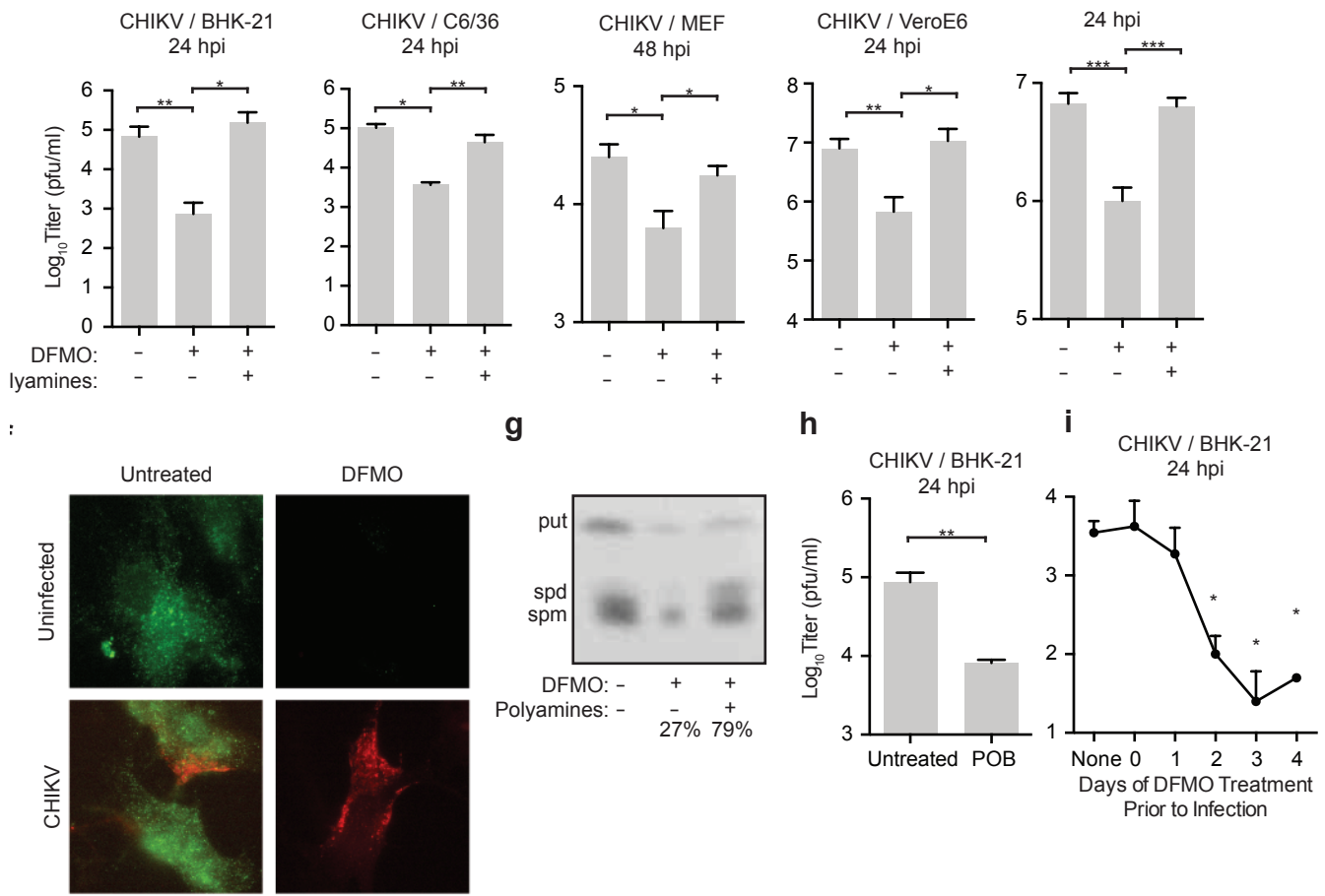


Figure 1

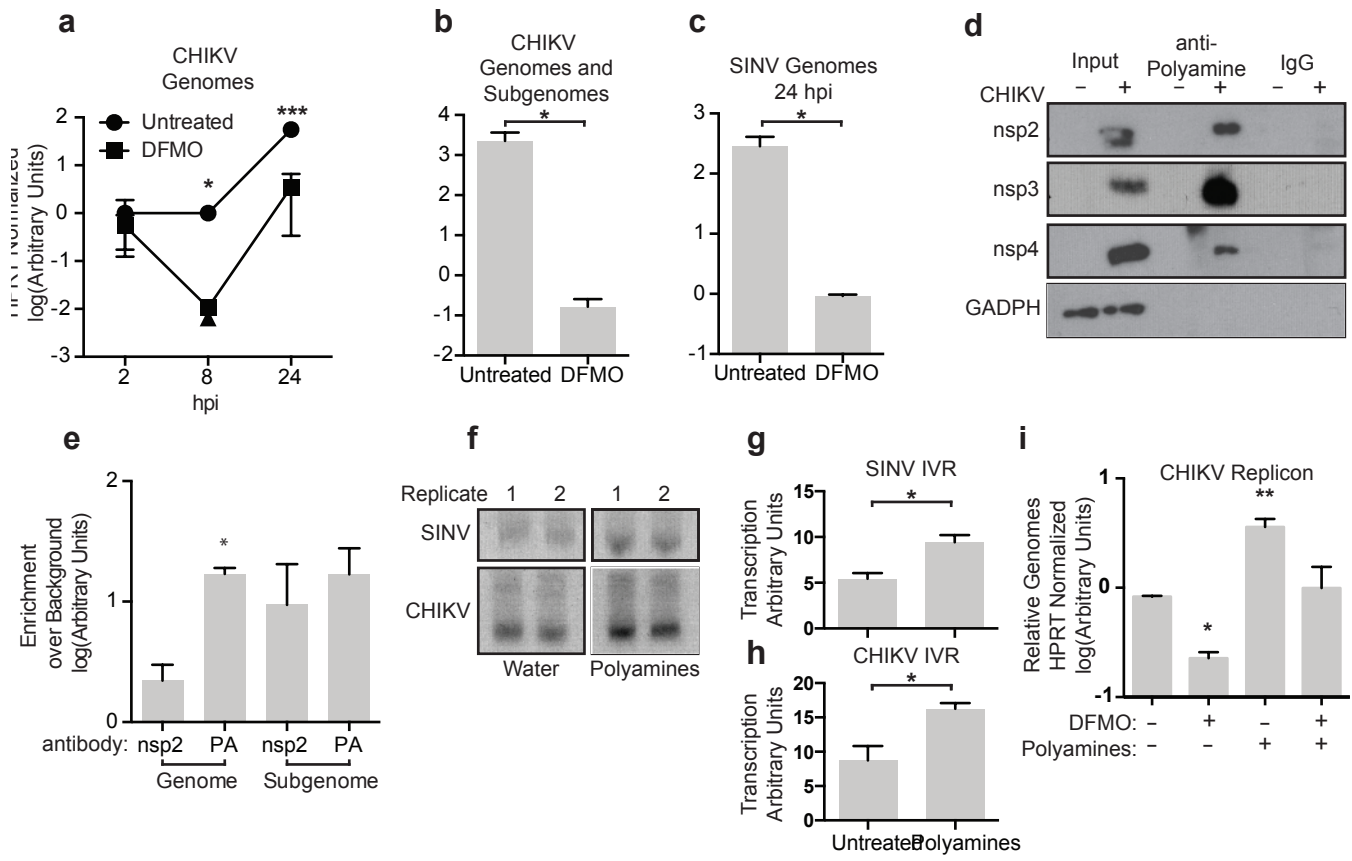


Figure 2

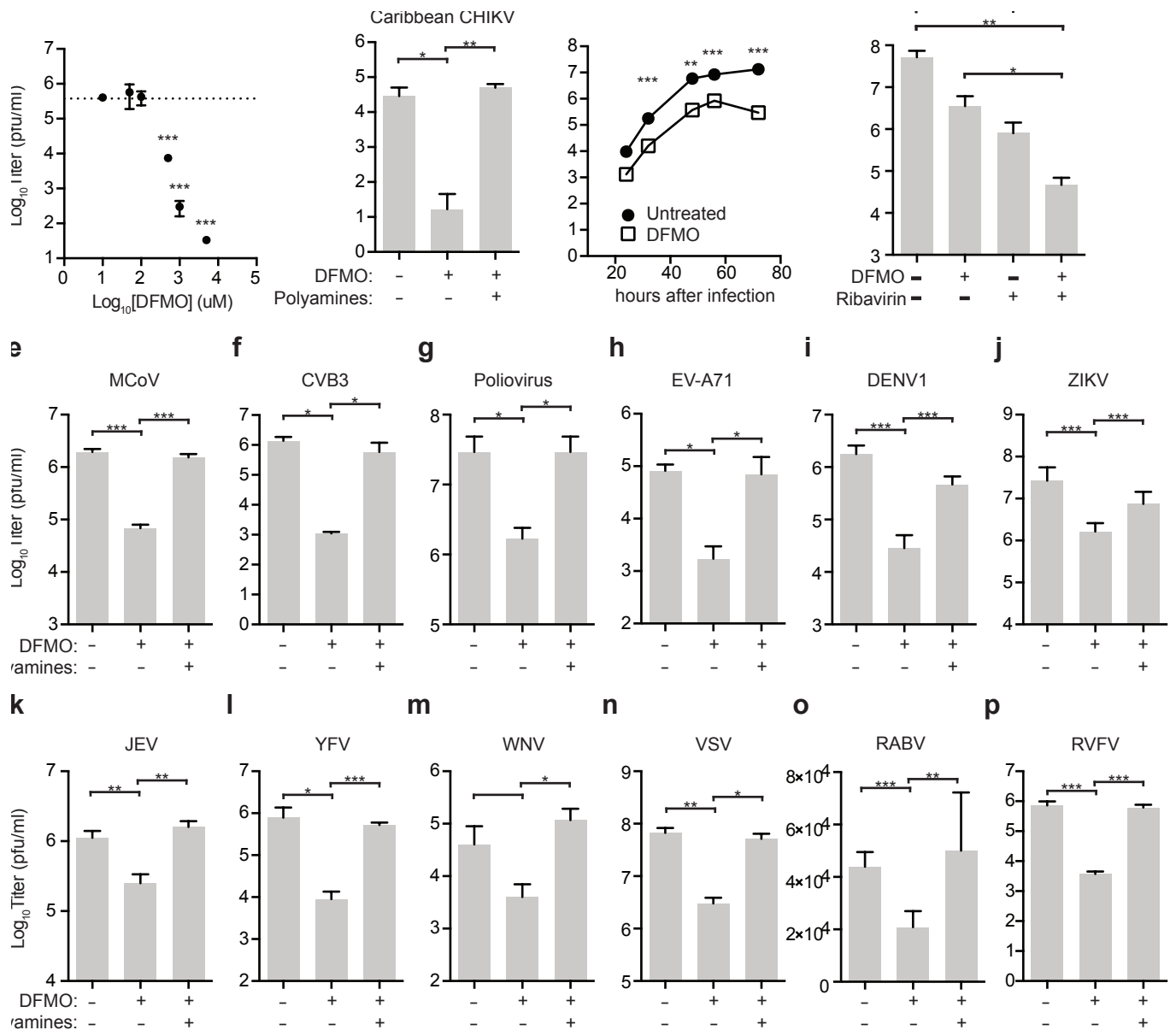


Figure 3