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Curcumin inhibits Zika and chikungunya virus infection by inhibiting cell binding

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ABSTRACT

Several compounds extracted from spices and herbs exhibit antiviral effects *in vitro*, suggesting potential pharmacological uses. Curcumin, a component of turmeric, has been used as a food additive and herbal supplement due to its potential medicinal properties. Previously, curcumin exhibited antiviral properties against several viruses, including dengue virus and hepatitis C virus, among others. Here, we describe the antiviral effect of curcumin on Zika and chikungunya viruses, two mosquito-borne outbreak viruses. Both viruses responded to treatment of cells with up to 5 μM curumin without impacting cellular viability. We observed that direct treatment of virus with curcumin reduced infectivity of virus in a dose- and time-dependent manner for these enveloped viruses, as well as vesicular stomatitis virus. In contrast, we found no change in infectivity for Coxsackievirus B3, a non-enveloped virus. Derivatives of curcumin also exhibited antiviral activity against enveloped viruses. Further examination revealed that curcumin interfered with the binding of the enveloped viruses to cells in a dose-dependent manner, though the integrity of the viral RNA was maintained. Together, these results expand the family of viruses sensitive to curcumin and provide a mechanism of action for curcumin's effect on these enveloped viruses.

1. Introduction

The development of novel antiviral compounds presents several challenges and requires significant amounts of effort for drug design and validation. Therefore, exploring the repurposing of already-approved pharmaceuticals or the use of natural compounds can provide alternatives to the development of novel antivirals. Curcumin, a component of turmeric, has been described to have several functions in preventing or treating diseases, including cancers and viral infections (Shome et al., 2016). Curcumin has several effects on treated cells, such as alteration of NF-κB signaling (Singh and Aggarwal, 1995) and autophagy (Shinojima et al., 2007), among others, and the availability of curcumin, especially in the diet, highlights the potential of this molecule in the treatment of disease.

Previous work has demonstrated that curcumin is an antiviral compound, with activity against diverse viruses such as dengue virus (serotype 2) (Padilla-S et al., 2013), herpes simplex virus (Kutluay et al., 2008), and human immunodeficiency virus (Mazumder et al., 1995), among others. Diverse mechanisms have been suggested as to how curcumin impacts each of these viruses, and, thus, curcumin likely has multiple effects on viruses and the cells they infect. Exploration of these phenotypes and the diversity of viruses that are affected by curcumin will highlight the potential of this compound in the treatment of viral infection, especially for emerging or re-emerging pathogens.

Zika and chikungunya viruses are arthropod-borne viruses (arboviruses), which transmit through a mosquito vector. Zika virus (ZIKV), a flavivirus, and chikungunya virus (CHIKV), an alphavirus, are both positive-stranded, enveloped RNA viruses that replicate within insect and human hosts and can cause severe disease. The devastating effects of CHIKV as it spread from Africa to Southeast Asia to the Americas were an ominous warning for the potential spread of relatively-unknown viruses worldwide (Weaver and Lecuit, 2015). The clinical symptoms of CHIKV range from asymptomatic cases to long-lasting arthritis, which is often debilitating (Couderc and Lecuit, 2015). When ZIKV began to spread, again from Africa to Southeast Asia to the Americas, the impact of the virus was similarly devastating, especially given the newly-observed links

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between the virus and neurological conditions and microcephaly (Shuaib et al., 2016). The persistent threat of viruses such as CHIKV and ZIKV necessitate measures, including the development or repurposing of antiviral compounds, to prepare for future outbreaks.

Here, we report the effects of curcumin on ZIKV and CHIKV *in vitro*, demonstrating for the first time the antiviral activity of curcumin against ZIKV. Similar to phenotypes observed for other viruses, curcumin inhibited ZIKV and CHIKV replication, most potently when treatment of cells was performed prior to infection. Coxsackievirus B3, a nonenveloped enterovirus, was insensitive to curcumin, however. We further observed that CHIKV and ZIKV lost infectivity when incubated directly with curcumin or derivatives of curcumin, suggesting that curcumin alters the ability of the virus to infect cells. In fact, we find that curcumin reduces viral replication by inhibiting viral binding at the cell surface. Together, these results demonstrate antiviral activity of curcumin against ZIKV and CHIKV via the inhibition of binding. Given the abundance of curcumin and its prevalence in the human diet, the compound holds significant promise for treatment of viral infection.

2. Materials and methods

2.1. Cell culture

HeLa, BHK-21, and Vero-E6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with newborn calf serum (NBCS; Sigma-Aldrich) and penicillin-streptomycin (Life Technologies). Cells were maintained at 37 °C in 5% CO_2.

2.2. Drug treatment

Curcumin (Sigma-Aldrich) was diluted to 10 mM in DMSO and further diluted to 100 μM in PBS. Analogs of curcumin, demethoxycurcumin, bisdemethoxycurcumin, EF-24, and FLLL31 (Sigma-Aldrich) were similarly diluted in DMSO. Cells were treated with curcumin or analogs by direct addition of the diluted compound to the media. Cells were pretreated with curcumin or analogs 2 h prior to infection (or as indicated), and compounds were maintained on the cells throughout infection.

For direct incubation of virus with curcumin and analogs, viruses were combined with serum-free DMEM containing curcumin (or analog) to the indicated concentration. The mixture was then incubated at 37 °C for 8 h prior to plaque assay to determine titers of virus surviving treatment. Viruses were similarly incubated in serum-free DMEM without drug. Titers of virus incubated with drug were compared to titers from virus without drug to compute the percent surviving treatment.

2.3. Viability assays

Cells were cultured as described, with escalating concentrations of curcumin. After 48 h of treatment, cells were assayed for viability using trypan blue exclusion and cell counting. In addition, cells were assayed for ATP levels using the CellTiter-Glo reagent (Promega) according to the manufacturer's specifications. For curcumin analog treatment, viability was measured 24 h after treatment. A standard curve for ATP concentration was made using 10-fold serial dilutions of ATP, from 1 μM to 100 mM. All measured samples were found to be within the linear range and [ATP] was calculated from the linear regression equation from the linear portion of the standard curve.

2.4. Infection and enumeration of viral titers

CHIKV (La Réunion strain, 06-049 AM258994) and VSV (Indiana strain) were derived from passage of virus in BHK-21 cells. CVB3 (Nancy strain) was derived from the first passage in HeLa cells. ZIKV (HD78788 strain; Faye et al., 2014) was derived from the third passage of virus in C6/36 cells. Viral stocks were maintained at −80 °C.

For infection, virus was diluted in serum-free DMEM for a multiplicity of infection (MOI) of 0.1, 1 or 10. Viral inoculum, containing curcumin where appropriate, was overlaid on cells for 30 m to 1 h, and virus was then cleared from the cells and the cells were washed three times with PBS. Medium containing curcumin as indicated was replenished.

Supernatants were collected from CHIKV and ZIKV infections at 48 hpi, unless otherwise indicated. Supernatant from CVB3-infected cells were collected at 24 hpi. Titrations were performed as previously described (Mounce et al., 2016a,b). Briefly, dilutions of cell supernatant or mixture of incubated virus/drugwere prepared in serum-free DMEM and used to inoculate confluent monolayers of Vero-E6 cells for 30 m to 1 h at 37 °C. Cells were then overlaid with 0.8% agarose in DMEM containing 1.6% NBCS. After 24 h incubation for VSV, 48 h for CVB3, 72 h for CHIKV, or 96 h for ZIKV, cells were fixed with 4% formalin and revealed with crystal violet solution (10% crystal violet [Sigma-Aldrich], 20% ethanol). Plaques were enumerated and used to back-calculate the number of plaque-forming units (pfu) per milliliter of collected volume.

2.5. RNA extraction and genome quantitation

For cell culture experiments and binding assay, media was cleared from cells and Trizol reagent (Sigma-Aldrich) directly added. For quantitation of viral genomes after virus incubation with curcumin or analogs, Trizol was added to virus-compound mixes in equal volume. RNA was purified according to the manufacturer's protocol. Purified RNA was DNase treated (Ambion) and subsequently used for cDNA synthesis using Maxima H Minus reverse transcriptase (Life Technologies), according to the manufacturer's protocol, with 500 ng of RNA and random hexamer primers.

Following cDNA synthesis, qRT-PCR was performed using StepOnePlus (Applied Biosystems, Norwalk, CT) and SYBR green mastermix (Life Technologies) as previously described (Mounce et al., 2016a,b). Samples were held at 95° for 30s prior to 40 cycles of 95° for 10s and 60° for 15s. Primers for CHIKV targeted residues 8625-8727 in the viral genome/subgenome (F: 5′-CCA-TAG-TCC-CGT-AGC-ACT-AGA-A-3′, R: 5′-GTG-GCT-GTC-ATC-CGT-CTT-TAT-T-3′). Primers for ZIKV targeted NS5 (F:5′-AAA-TAC-ACA-TAC-CAA-AAC-AAA-GTG-GT-3′; R: 5′-TCCACTCCCTCTCTGGTCTTG-3′). Primers for CVB3 targeted residues 6914-6983 in the viral genome (F: 5′-GAT-CGC-ATA-TGG-TGA-TGA-TGT-GA-3′; R: 5′-AGC-TTC-AGC-GAG-TAA-AGA-TGC-A-3′). Primers against GAPDH (F: 5′-TGT-GAT-GGG-TGT-GAA-CCA-CGA-GAA-3′, R: 5′-GAG-CCC-TTC-CAC-AAT-GCC-AAA-GTT-3′) were used to normalize to total RNA using the ΔCT method.

2.6. Western blot analysis

Cells were treated with 5 μM curcumin prior to and after infection with ZIKV and CHIKV. At 24 hpi, cells were washed once in PBS and then collected in XT Sample Buffer (Bio-Rad), containing 10% betamercaptoethanol. Samples were boiled for 10 min and then run on a 4–12% gradient NuPAGE gel (ThermoFisher). Gel was transferred onto a nitrocellulose membrane using the iBlot 2 (ThermoFisher). The membrane was probed using the iBind Flex (ThermoFisher) with antibodies against CHIKV nsP1 (1:1000) and

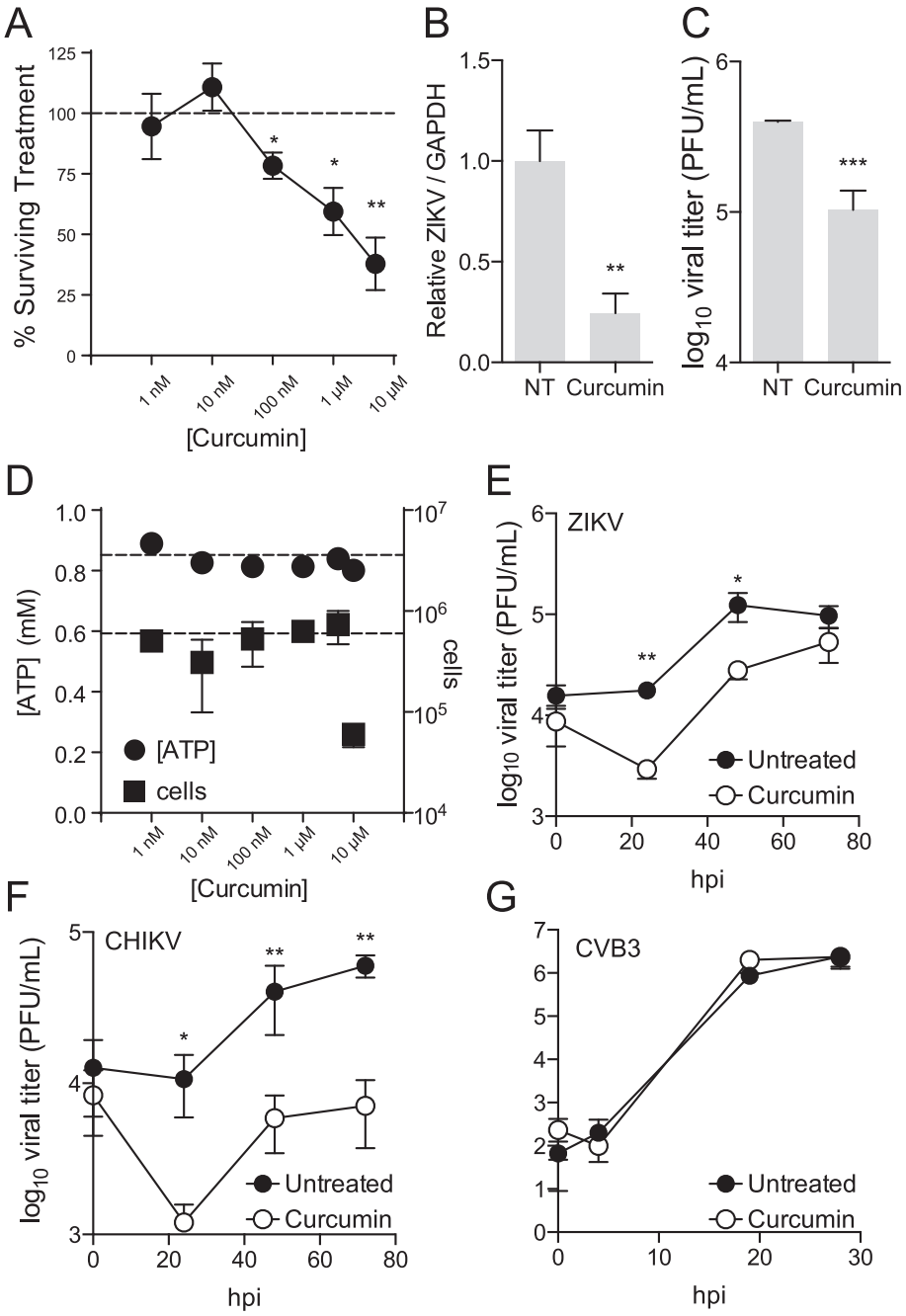

Fig. 1. Curcumin inhibits Zika and chikungunya viruses. (A) HeLa cells were treated with escalating doses of curcumin and infected with ZIKV at MOI 0.1 2 h later. Viral titers were determined at 48 hpi and compared to untreated controls to determine the percent surviving treatment (titer in treated cells divided untreated cells multiplied by 100%). (B) ZIKV genomes were quantified after treatment with 5 μM curcumin. (C) ZIKV titers in BHK-21 cells treated with 5 μM curcumin for 2 h prior to infection. Titers were determined at 48 hpi. (D) HeLa cells were treated with escalating doses of curcumin and cellular viability measured 48 h later by counting cells (squares) and determining ATP content (circles). Horizontal lines represent cell count (above) and ATP content (below) of untreated cells. HeLa cells were treated with 5 μM curcumin for 2 h prior to infection with ZIKV (E), CHIKV (F), or CVB3 (G). Viral titers were determined at the indicated times. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ using a one-tailed Student's T-test ($N \geq 3$). Error bars represent one standard error of the mean. NT, untreated (control).

ZIKV NS1 (1:500), kindly provided by Marie Flamand, and secondary anti-rabbit antibodies (1:2000). Gel was visualized by myECL Imager (ThermoFisher).

2.7. Binding assay

Virus was incubated with escalating doses of curcumin. Following 4 h of incubation at 37 °C, 300 μL of virus was added to confluent HeLa cells in 12-well plates, and virus inoculum incubated on the cells for 1 h at 4 °C. Cells were subsequently washed five times with PBS and cells with bound virus were then collected in Trizol reagent. RNA was purified and analyzed for viral genomes as described above.

2.8. Transfection and luciferase assays

HeLa cells were treated with 5 μM curcumin or 200 μM azacytidine for 2 h and subsequently transfected using Lipofectamine

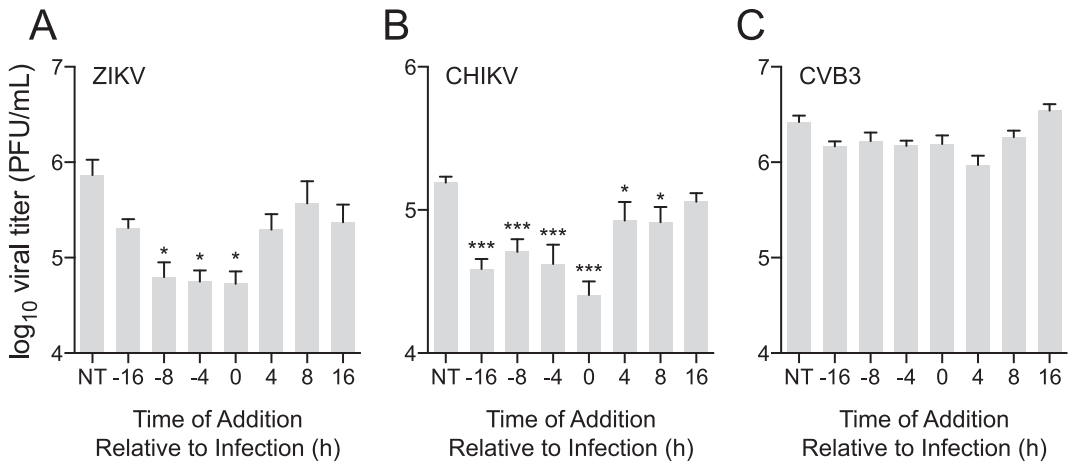

Fig. 2. Curcumin inhibits Zika and chikungunya viruses with pretreatment of cells. HeLa cells were treated with 5 μM curcumin at the indicated times relative to infection at MOI 0.1 with (A) ZIKV, (B) CHIKV, and (C) CVB3. Titers were determined at 48 hpi for ZIKV and CHIKV and 24 hpi for CVB3. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ using a one-tailed Student's T-test ($N \geq 3$). Error bars represent one standard error of the mean. NT, untreated (control).

2000 (Life Technologies) with CHIKV replicon RNA (Fros et al., 2010), CVB3 genomes, or siCHECK luciferase construct. For luciferase assays, cells were harvested via trypsin 24 h post transfection. Luciferase assays were performed according to the manufacturer's recommendations (Promega) and were measured using a Wallac 1420 instrument (Perkin Elmer).

2.9. Statistical analysis

Prism 6 (GraphPad) was used to generate graphs and perform statistical analysis. A one-tailed Student's T-test was used to compare groups, with $\alpha = 0.05$.

2.10. Structure drawings

Structures of curcumin and analogs were generated in BKChem.

3. Results

3.1. Curcumin inhibits replication of Zika and chikungunya viruses

As a test of the antiviral activity of curcumin, we treated HeLa cells with escalating doses of curcumin, from 1 nM to 5 μM for 2 h prior to infection with ZIKV, at a multiplicity of infection of 1. At 48 h post infection (hpi), supernatant was collected and titered. We observed a dose-dependent decrease in viral titers, with concentrations at or above 100 nM being statistically significantly decreased compared to untreated controls (as indicated by the dashed line, Fig. 1A). The decrease in ZIKV titers was further corroborated by measuring virus-specific RNA. Cell-associated virus was collected at 48 hpi and viral genomes were quantified via qRT-PCR. We observed a reduction in ZIKV genomes with 5 μM curcumin treatment (Fig. 1B). Similar to the results observed in HeLa cells, we found that pretreatment of BHK-21 cells with 5 μM curcumin 2 h prior to infection similarly reduced ZIKV titers at 48 hpi (Fig. 1C), indicating that curcumin is effective in distinct cell types. Because curcumin has been described to lead to apoptosis (Kuo et al., 1996; Samaha et al., 1997), we measured cellular viability in HeLa cells similarly treated with escalating doses of curcumin. By cell count and ATP content measures, we observed a marked decrease in cellular viability at 10 μM doses (Fig. 1C). Thus, all assays were performed with 5 μM curcumin to mitigate the effects of cellular viability on viral titers.

To begin deciphering how curcumin could impact ZIKV replication, we performed a time course of infection. HeLa cells were pretreated for 2 h with 5 μM curcumin and infected at an MOI of 1. Viral titers were determined every 24 hpi. We noted that curcumin treatment resulted in significantly decreased titers at 24 and 48 hpi, though ZIKV recovered to untreated levels by 72 hpi (Fig. 1E). In a similar vein, pretreated HeLa cells were infected with CHIKV and titers determined every 24 hpi. Again, we observed significantly decreased viral titers in curcumin-treated cells compared to untreated controls (Fig. 1F).

Previous studies had suggested that enveloped viruses are specifically inhibited by curcumin (Chen et al., 2013). Thus, we investigated whether Coxsackievirus B3 (CVB3), a non-enveloped enterovirus, was sensitive to curcumin. Over a course of infection, we observed no significant differences in CVB3 titers in curcumin-treated cells compared to untreated controls (Fig. 1G). Together, these results suggest that curcumin is effective against ZIKV and CHIKV, both enveloped viruses, but not CVB3. Further, these results highlight that the cells treated with 5 μM curcumin are viable, as in Fig. 1D, and capable of producing virus.

3.2. Curcumin inhibits ZIKV and CHIKV with pretreatment

To further understand the mechanism whereby curcumin could affect viral replication, we performed time-of-addition experiments with ZIKV, CHIKV, and CVB3. HeLa cells were treated with 5 μM curcumin at various times before and after infection and viral titers were determined at 48 h after infection at MOI 0.1 with CHIKV and ZIKV, or 24 h after infection with CVB3, after several rounds of viral replication. We noted that both ZIKV and CHIKV were sensitive to curcumin, even when treatment was initiated after infection (Fig. 2A and B); however, treatment was most effective when performed prior to or at the time of infection. As we noted earlier, curcumin did not have an impact on CVB3 replication regardless of the time of addition (Fig. 2C). These results suggest that curcumin's antiviral effect is early in infection, potentially prior to the onset of viral replication.

3.3. Curcumin does not affect RNA or protein accumulation

Decreased ZIKV and CHIKV titers even when drug was added after infection could mean that curcumin is affecting viral infection at the time of binding/entry because the virus has several rounds of

entry, replication, and egress within in 48 h. However, this result could also suggest that additional viral processes, such as RNA replication or protein expression, are affected by curcumin. To differentiate these two possibilities, we infected cells at high MOI of 10 to ensure that all cells were infected to prevent multiple rounds of infection, treated cells with curcumin at times before and after infection, and collected at 24 hpi to analyze for viral RNA and protein. When we compared intracellular RNA, we found that for both ZIKV and CHIKV, viral RNA levels were significantly decreased only when cells were pretreated (Fig. 3A and B). Even when cells were treated with curcumin immediately following incubation with the inoculum (time of addition of 0 h), no changes could be seen in intracellular viral RNA levels. Similarly, when we looked at viral RNA in the supernatant (progeny genomes), we found that only pretreatment had an effect on CHIKV and ZIKV genomes (Fig. 3C and D). These results suggest that treatment of cells with curcumin after viral entry has proceeded does not impact viral RNA accumulation.

We also investigated whether viral protein synthesis was impacted by looking at nonstructural protein levels for ZIKV and CHIKV via western blot. As with RNA accumulation, ZIKV NS1 and CHIKV nsP1 protein levels were decreased only with pretreatment (Fig. 3E). Again, treatment of cells after viral binding and entry did not impact protein accumulation. Together, these results suggest that curcumin does not impact viral RNA or protein accumulation and the effect of curcumin on viral replication is prior to the onset of these processes, possibly at the level of binding or entry.

3.4. Curcumin reduces infectivity of enveloped viruses in a dose-dependent manner

Because curcumin has an impact early in infection and because curcumin is a lipophilic molecule, we investigated whether curcumin may be reducing the infectivity of viruses. To this end, we incubated viruses with increasing doses of curcumin, from 1 μM to 1 mM at 37 °C for 8 h. Incubated viruses were subsequently diluted and directly titered, without infection. We observed that CVB3 was insensitive to direct curcumin treatment, as no changes in viral titer were observed, even at the 1 mM dose (Fig. 4A, black squares). In contrast, both ZIKV and CHIKV were sensitive to curcumin treatment, losing greater than 99% of their infectivity at the 1 mM dose (white and gray circles). We additionally used vesicular stomatitis virus as an additional enveloped virus, and we noted a significant decrease in viral infectivity with increased curcumin treatment (black circles).

To explore the time-dependence of this phenotype, ZIKV was incubated with 5 μM curcumin and samples were collected every 2 h and titered. Compared to untreated controls, curcumin-treated virus lost infectivity with as little as 2 h of incubation (Fig. 4B), suggesting that curcumin interferes with viral infectivity when incubated directly with virus in a time- and dose-dependent manner.

3.5. Curcumin structural analogs reduce infectivity of enveloped viruses

The chemical structure of curcumin can be manipulated in several ways to form derivative compounds with potentially novel functions (Fig. 5A). To determine whether these compounds exhibited antiviral activity, we incubated ZIKV, CHIKV, CVB3, and VSV with escalating doses of demethoxycurcuming (Fig. 5B), bisdemethoxycurcumin (Fig. 5C), EF-24 (Fig. 5D), and FLLL31 (Fig. 5E). We found that while each of these compounds exhibited antiviral activity against the enveloped viruses but not CVB3, both demethoxy- and bisdemethoxycurcumin exhibited inhibition levels

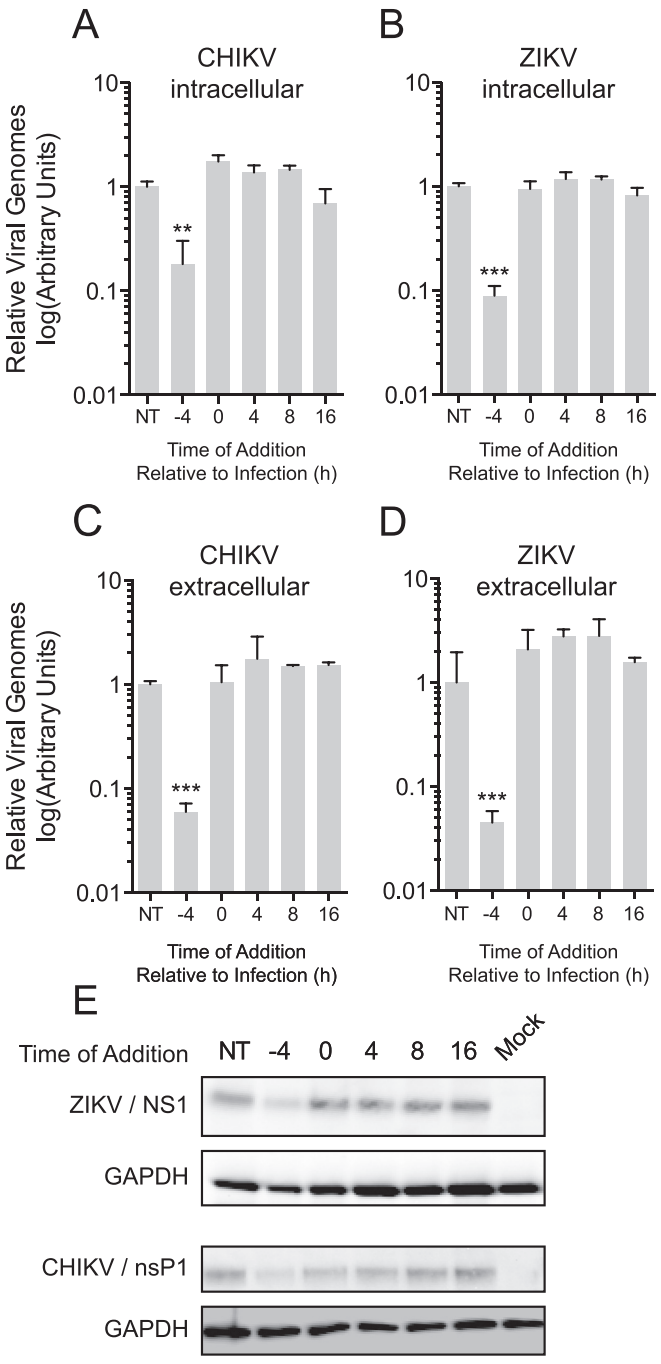

Fig. 3. Curcumin does not impact viral RNA or protein accumulation if treatment is initiated after infection. HeLa cells were treated with 5 μM curcumin prior to, immediately after, or hours after infection with CHIKV and ZIKV at MOI 10. At 24 hpi, cells were harvested and intracellular genomes quantified by qRT-PCR for (A) CHIKV and (B) ZIKV. Extracellular genomes were similarly quantified for (C) CHIKV and (D) ZIKV. Viral protein accumulation of nonstructural protein 1 was analyzed by western blot for ZIKV (NS1 antibody, above) and CHIKV (nsP1 antibody, below). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ using a one-tailed Student's T-test ($N \geq 3$). Error bars represent one standard error of the mean. NT, untreated (control).

similar to unmodified curcumin. Both EF-24 and FLLL31 had limited antiviral activity, though CHIKV and VSV titers were reduced by up to 90% when treated with 50 or 500 μM doses of the compounds. We also measured whether these compounds exhibited cellular toxicity by incubating HeLa cells with escalating doses and measuring ATP levels of the cells. While both demethoxy- and

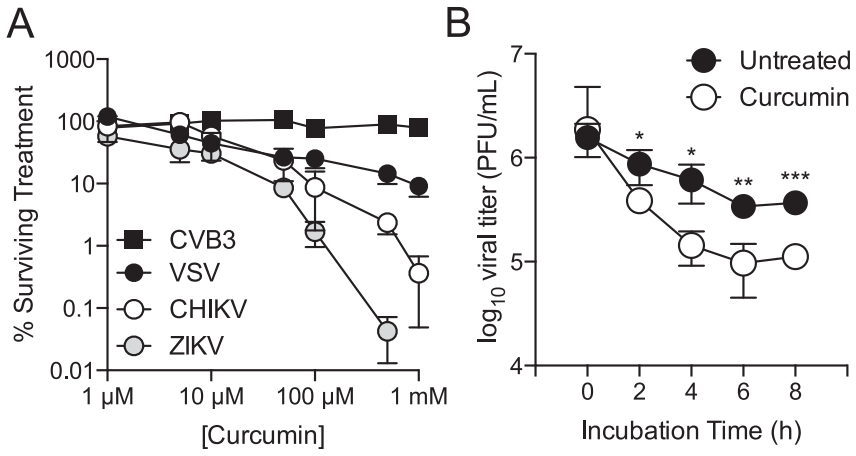

Fig. 4. Enveloped viruses are sensitive to curcumin in a dose-dependent manner. (A) ZIKV, CHIKV, VSV, and CVB3 were incubated with escalating doses of curcumin at 37 °C for 8 h prior to immediate titration on Vero cells. (B) ZIKV was incubated with or without 5 μM curcumin for the indicated times and immediately titrated on Vero cells. Percent surviving treatment was calculated by comparing titers from virus incubated with drug compared to untreated virus. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 using a one-tailed Student's T-test (N ≥ 3). Error bars represent one standard error of the mean.

bisdemethoxycurcumin exhibited toxicities similar to unmodified curcumin, namely marked decrease in viability at 10 μM doses, both EF-24 and FLLL31 exhibited reduced viability at 5 μM. Thus, within limits, modification of curcumin maintains antiviral activity, specifically when modifying the methoxy groups of the molecule. Calculated 50% inhibitory concentration (IC50) values were below

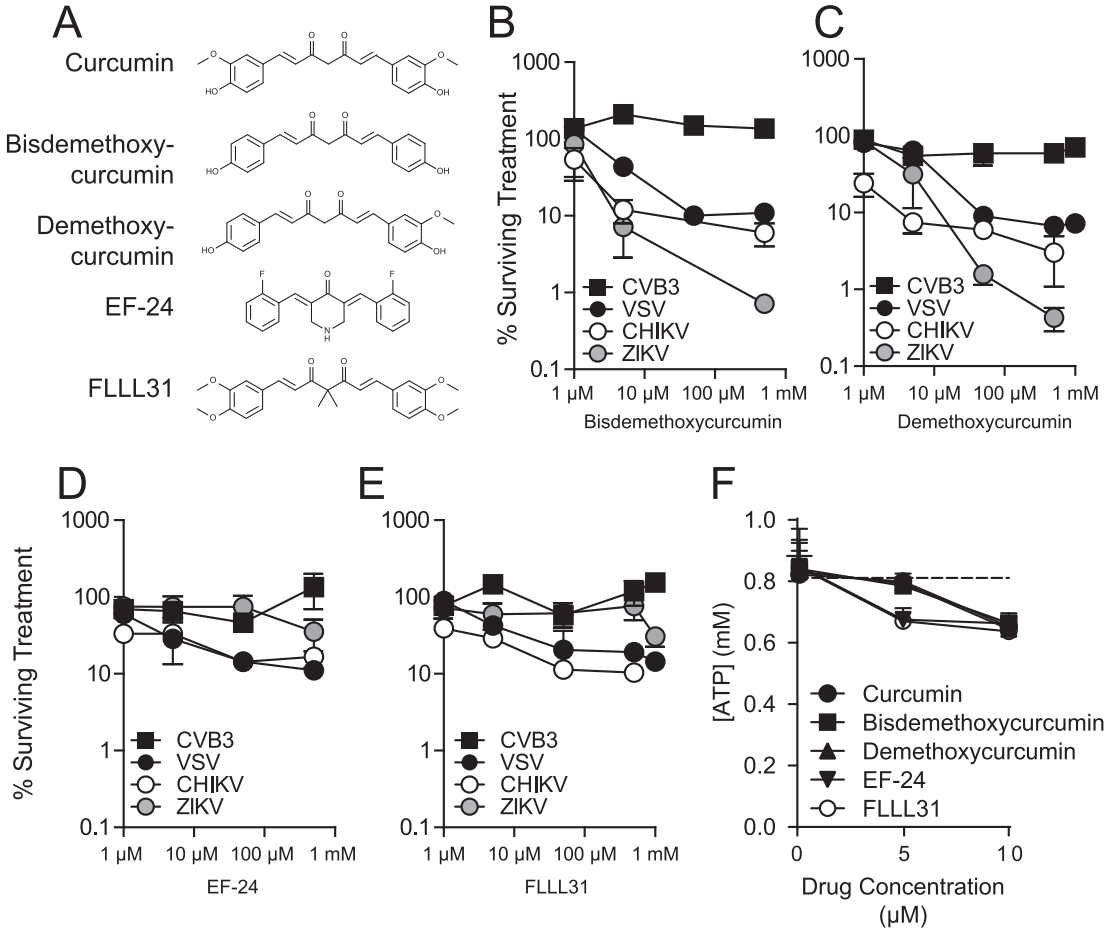

Fig. 5. Curcumin structural analogs reduce infectivity of enveloped viruses. (A) Chemical structures of curcumin and the analogs bisdemethoxycurcumin, demethoxycurcumin, EF-24, and FLLL31. CHIKV (white circles), ZIKV (gray circles), VSV (black circles), and CVB3 (black squares) were incubated with escalating doses of (B) bisdemethoxycurcumin (C) demethoxycurcumin, (D) EF-24, and (E) FLLL31 for 8 h prior to immediate titration on Vero cells. Percent surviving treatment was calculated by comparing titers from virus incubated with drug compared to untreated virus. (F) HeLa cells were incubated with increasing doses of curcumin and analogs and ATP content measured 24 h later. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 using a one-tailed Student's T-test (N ≥ 3). Error bars represent one standard error of the mean.

Table 1
50% inhibitory concentrations (IC50) and cytotoxicity concentrations (CC50) of compounds and viruses used in these studies.

	Virus	Curcumin	Bisdemethoxycurcumin	Demethoxycurcumin	EF-24	FLLL31
IC50 (μM)	ZIKV	1.90	3.61	5.91	1.49	6.85
	CHIKV	3.89	4.84	0.89	13.6	7.92
	VSV	4.98	4.94	5.41	4.06	4.14
	CVB3	94.5	81.9	44.3	63.9	33.1
CC50 (μM)		11.6	16.0	13.2	3.46	3.53

the 50% cytotoxicity concentration (CC50) values for curcumin, bisdemethoxycurcumin, and demethoxycurcumin, but not EF-24 or FLLL31 (Table 1).

3.6. Curcumin blocks binding of enveloped viruses to cell surface

Having observed reduced titers by incubating virus with curcumin, we hypothesized that curcumin was directly interfering with the viruses' ability to bind to the cell surface. To investigate this, we treated viruses with 1, 10, or 100 μM curcumin, and then used this virus to inoculate confluent monolayers of HeLa cells. Following an hour-long incubation, the cells were thoroughly washed to remove inoculum. Cells and attached virus was then collect and analyzed for bound viral genomes via qRT-PCR. For both CHIKV and ZIKV, we noted a dose-dependent decrease in bound virus (Fig. 6A and B). In contrast, CVB3 showed no alteration in its ability to bind cells (Fig. 6C). This decrease in cell-associated virus was not due to a general degradation of viral genomes with curcumin treatment, however, because total viral genomes within the inoculum was not altered with curcumin treatment for any virus (Fig. 6D–F). We also observed this phenotype when incubating CHIKV or ZIKV directly with the curcumin analogs at a concentration of 1 μM (Fig. 6G, H), but these analogs did not impact CVB3 infectivity (Fig. 6I).

3.7. Transfection of virus bypasses antiviral activity of curcumin

To determine whether we could bypass curcumin's effect on viral infectivity, we transfected curcumin-treated HeLa cells with a replicon encoding the CHIKV nonstructural replication complex proteins, as well as luciferase. By measuring luciferase activity coming from this transfected construct, we found no significant differences in replication of the CHIKV replicon in untreated or curcumin-treated cells, further implicating the steps in viral entry as the target of curcumin's activity (Fig. 7A). As a control, we treated cells with azacytidine (AZC), a nucleoside analog and known antiviral, and we observed significantly reduced luciferase activity, verifying that the CHIKV construct was sensitive to an antiviral. Similarly, we transfected cells with CVB3 RNA and viral titers measured 24 h later. No significant differences in titer were observed with curcumin treatment, though the virus remained sensitive to AZC (Fig. 7B). To verify that transfection efficiency was similar wiith curcumin treatment, we also transfected a plasmid (siCHECK) encoding luciferase, in which we observed no differences in luciferase activity with either curcumin or AZC treatment (Fig. 7C). These data suggest that curcumin does not interfere directly with CHIKV replication machinery and that curcumin's antiviral activity likely derives from its inhibition of virus-cell binding.

4. Discussion

The ability of a virus to engage its cellular receptor, enter the cell, and replicate is a complex process that affords many opportunities for the development of antiviral strategies. Here, we describe how enveloped viruses can be inhibited through the compound curcumin, a component of turmeric, and that curcumin acts to inhibit virus binding to the cell surface. When the enveloped viruses CHIKV, ZIKV, or VSV were incubated with curcumin, their ability to bind to cell surfaces was degraded and their infectivity was lost. Interestingly, these same effects were not seen with CVB3, a non-enveloped virus, even at very high doses of curcumin. Together, our results expand the number of viruses that are sensitive to curcumin and highlight the mechanism by which curcumin acts to inhibit ZIKV and CHIKV replication.

Previous reports have suggested that curcumin mediates its antiviral activity through diverse mechanisms. For instance, curcumin inhibits Japanese encephalitis virus via a dysregulated ubiquitin-proteasome system and an accumulation of ubiquitinated proteins (Dutta et al., 2009). Curcumin's disruption of NF-κB signaling inhibits Rift Valley fever virus replication (Narayanan et al., 2012). Hepatitis C virus is sensitive to SREBP-1, an important transcription factor in sterol synthesis regulation, which is inhibited by curcumin (Kim et al., 2010). Additionally, curcumin was shown to impact HCV replication through binding and fusion (Anggakusuma et al., 2014), similar to the results we have obtained for ZIKV and CHIKV. Curcumin can also directly affect the viral replication machinery, as HIV-1 integrase activity is reduced in the presence of curcumin (Mazumder et al., 1995). We reported that CVB3, a nonenveloped RNA virus, was insensitive to curcumin, either when added to cells or incubated directly with virus. Additionally, we saw no difference in CVB3 binding to the cell surface after curcumin treatment. Previous work had demonstrated that CVB3 was sensitive to curcumin, though at concentrations higher than we used in this study (Si et al., 2007). Enterovirus-71 (EV71), closely related to CVB3, was also demonstrated to be insensitive to curcumin, which was attributed to a lack of viral envelope (Chen et al., 2013), though curcumin could potentially inhibit viral replication at later steps with higher concentrations.

Precisely how curcumin inhibits virus binding to the cell surface remains unclear. Our data suggest that curcumin does not directly lead to the degradation of the viral RNA; thus, curcumin is not destroying viral particles. Instead, curcumin may be acting by altering the membranes of these enveloped viruses. Previous work with HCV demonstrated that curcumin affects membrane fluidity and, thus, binding and fusion of the virus to the cell surface (Anggakusuma et al., 2014). The changes in membrane fluidity may alter the conformation of viral glycoproteins, which are essential for the viruses to interact with the cells that they will infect. Because curcumin is a lipophilic molecule (Jaruga et al., 1998), it could interfere with receptor function by altering envelope dynamics on the virus itself. Also, curcumin may have effects on the host lipid membranes, which play several roles in viral infection, including entry. While previous results had suggested that curcumin alters transfection efficiency from cationic liposomes (Chen et al., 2013), we observed no alterations in transfection efficiency of curcumin-treated cells. Perhaps this difference could be due to different cell types or treatment regimens; nonetheless, our results suggest that curcumin does not affect ZIKV or CHIKV replication beyond the entry steps.

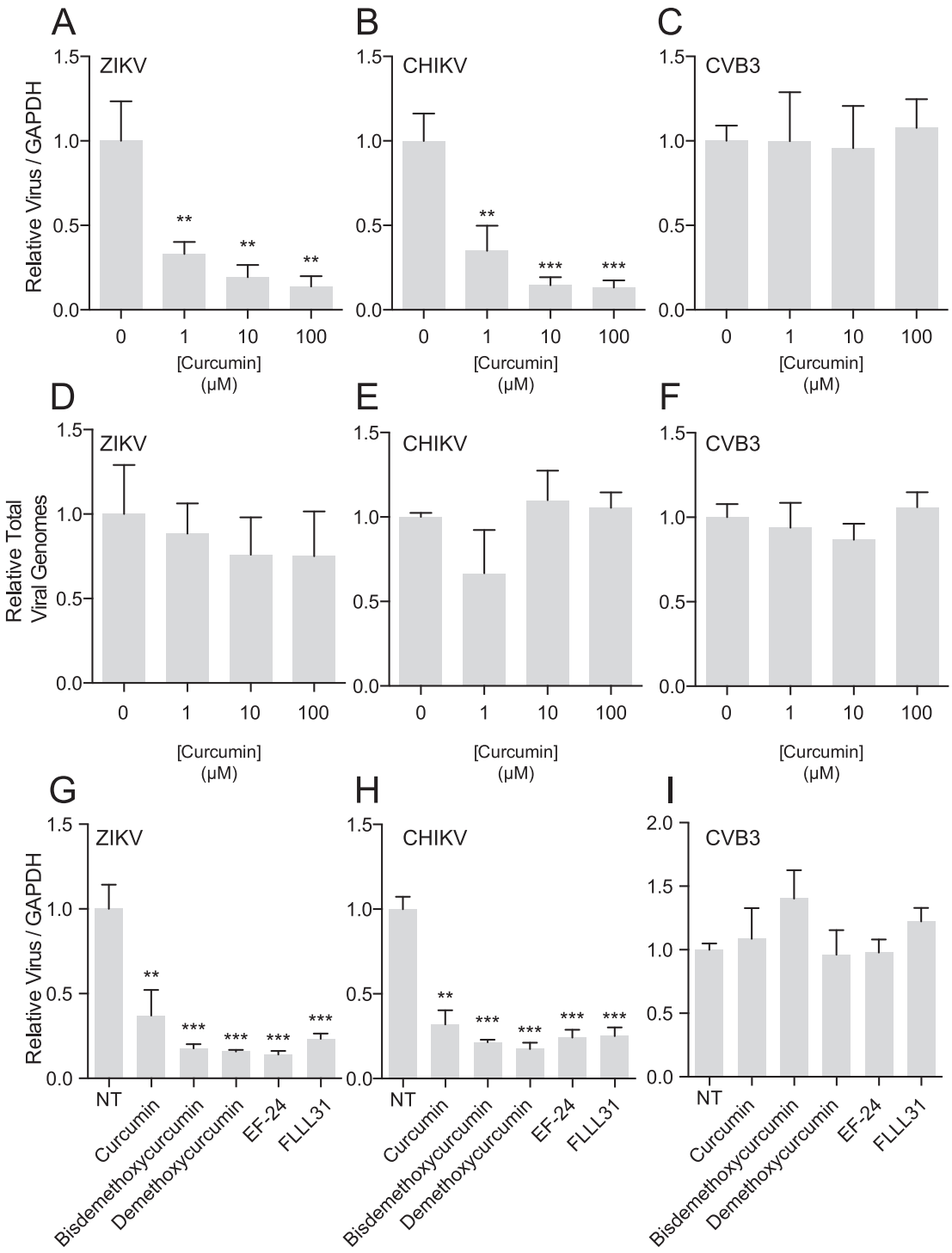

Fig. 6. Curcumin interferes with binding of enveloped viruses to cell surface. (A) ZIKV, (B) CHIKV, and (C) CVB3 were incubated with increasing doses of curcumin for 4 h prior to adhesion to HeLa cell surfaces for 1 h at 4 °C. Unbound virus was washed away and virus bound to the cell surface following this incubation was quantified using qRT-PCR with virus-specific primers. (D–F) Total viral genomes were quantitated after curcumin incubation without cellular adhesion for the same viruses. (G–I) ZIKV, CHIKV, and CVB3 were incubated with 1 μM curcumin or curcumin analog for 4 h prior to adhesion and analysis of surface binding as in panels A–C. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ using a one-tailed Student's T-test ($N \geq 3$). Error bars represent one standard error of the mean.

The ability of curcumin to prevent viral replication strongly suggests that this molecule and its derivatives may hold promise for the development of broad-range antivirals. Curcumin in the human diet, further, could provide a simple means to prevent infection by enveloped viruses. Due to the devastating effects of both ZIKV and CHIKV in regions where these viruses are emerging or re-emerging, novel pharmaceuticals are necessary to combat disease in infected individuals. Curcumin can be administered in a variety of ways, including orally (Kurita and Makino, 2013). In fact, oral administration of curcumin exhibits anticancer effects in mouse models. In a mouse model of cytomegalovirus, intragastric administration of curcumin significantly reduced viral DNA load

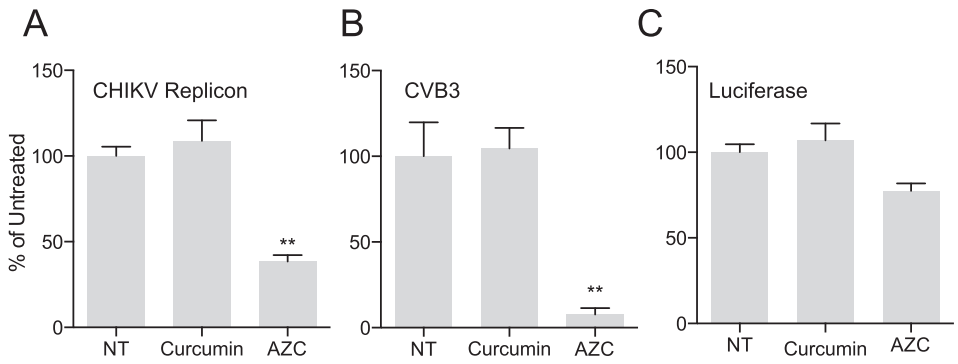

Fig. 7. Transfection of viral RNA bypasses the antiviral effect of curcumin. (A) RNA from a CHIKV replicon system, encoding luciferase, was transfected into HeLa cells pretreated with 5 μM curcumin or 200 μM azacytidine (AZC) for 2 h. Luciferase activity was measured 24 h later. (B) CVB3 RNA genomes were transfected into treated HeLa cells and viral titers determined 48 h later. (C) DNA plasmid encoding luciferase was transfected into treated HeLa cells and luciferase activity determined 24 h later. $^{**}p \leq 0.01$ using a one-tailed Student's T-test (N = 3). Error bars represent one standard error of the mean. NT, untreated (control).

and IgM levels, suggesting a potential for antiviral activity *in vivo* (Lv et al., 2014). However, work with HCV demonstrated no differences in viral replication in infected curcumin-treated versus untreated humanized mice (Anggakusuma et al., 2014). Thus, the delivery route, dose, and frequency of treatment require optimization for an effect *in vivo*. Given the relatively low toxicity of curcumin in normal, non-cancer cells, high doses can be used with few ill effects. Further, several clinical trials have been performed, demonstrating few side effects and some promising therapeutic results (Devassy et al., 2015). Poor bioavailability limits its potential, though several alternative formulations that may hold promise are under development (Anand et al., 2007). Additionally, derivatives of curcumin with enhanced activity and bioavailability could significantly enhance *in vivo* efficacy. In fact, a curcumin derivative exhibits activity against HIV-1 and has enhanced serum stability in a mouse model (Kumari et al., 2015). Altogether, curcumin holds significant promise in the treatment of enveloped virus infection, including outbreak viruses such as ZIKV and CHIKV.

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