Suppression of Zika Virus Infection and Replication in Endothelial Cells and Astrocytes by PKA Inhibitor PKI 14-22

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ABSTRACT The recent outbreak of Zika virus (ZIKV), a reemerging flavivirus, and its associated neurological disorders, such as Guillain-Barré (GB) syndrome and microcephaly, have generated an urgent need to develop effective ZIKV vaccines and therapeutic agents. Here, we used human endothelial cells and astrocytes, both of which represent key cell types for ZIKV infection, to identify potential inhibitors of ZIKV replication. Because several pathways, including the AMP-activated protein kinase (AMPK), protein kinase A (PKA), and mitogen-activated protein kinase (MAPK) signaling pathways, have been reported to play important roles in flavivirus replication, we tested inhibitors and agonists of these pathways for their effects on ZIKV replication. We identified the PKA inhibitor PKI 14-22 (PKI) to be a potent inhibitor of ZIKV replication. PKI effectively suppressed the replication of ZIKV from both the African and Asian/American lineages with a high efficiency and minimal cytotoxicity. While ZIKV infection does not induce PKA activation, endogenous PKA activity is essential for supporting ZIKV replication. Interestingly, in addition to PKA, PKI also inhibited another unknown target(s) to block ZIKV replication. PKI inhibited ZIKV replication at the postentry stage by preferentially affecting negative-sense RNA synthesis as well as viral protein translation. Together, these results have identified a potential inhibitor of ZIKV replication which could be further explored for future therapeutic application.

IMPORTANCE There is an urgent need to develop effective vaccines and therapeutic agents against Zika virus (ZIKV) infection, a reemerging flavivirus associated with neurological disorders, including Guillain-Barré (GB) syndrome and microcephaly. By screening for inhibitors of several cellular pathways, we have identified the PKA inhibitor PKI 14-22 (PKI) to be a potent inhibitor of ZIKV replication. We show that PKI effectively suppresses the replication of all ZIKV strains tested with minimal cytotoxicity to human endothelial cells and astrocytes, two key cell types for ZIKV infection. Furthermore, we show that PKI inhibits ZIKV negative-sense RNA synthesis and viral protein translation. This study has identified a potent inhibitor of ZIKV infection which could be further explored for future therapeutic application.

KEYWORDS Zika virus, inhibitor, PKI 14-22, PKA, endothelial cells, astrocytes

Zika virus (ZIKV) is an arthropod-borne virus transmitted mainly through the bites of infected *Aedes* mosquitoes in tropical and subtropical regions across the globe. ZIKV was first isolated in the Zika Forest in Uganda in 1947 (1). Outbreaks of ZIKV were reported in 2007 in Yap Island, Micronesia, and more recently in French Polynesia (2013 and 2014) (2, 3). Two ZIKV strains, the African and Asian strains, have been described, though some reports prefer the distinction between East and West African strains (4, 5). In 2015, ZIKV was detected in Brazil and was associated with a significant increase in the

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number of microcephaly cases in newborns (6–11). The strain detected in Brazil, which later spread in Latin America, was classified as the Asian strain (6). In February 2016, the World Health Organization (WHO) declared ZIKV infection to be a public health emergency of international concern (12). As of March 2016, 18 international companies and institutions were developing vaccines against ZIKV. Nevertheless, a vaccine is unlikely to be widely available for another 10 years. At the current stage, the search for an effective antiviral drug is still an urgent task around the globe.

ZIKV belongs to the *Flaviviridae* family and the *Flavivirus* genus and is thus closely related to dengue virus (DENV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), and West Nile virus (WNV) (13). Like other flaviviruses, ZIKV is enveloped with an icosahedral capsid and has a nonsegmented, single-stranded, 10-kb positive-sense RNA genome. The positive-sense RNA genome of ZIKV can be directly translated into viral proteins, including three structural proteins, i.e., the capsid (C), precursor of membrane (prM), and envelope (E) proteins, and seven nonstructural (NS) proteins, i.e., NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5. Previous studies have revealed the broad cellular tropism of ZIKV and the nature of cellular receptors that mediate ZIKV entry (14). Following the attachment of ZIKV to the host cell, the E glycoprotein binds to the endosomal membrane of the host cell to initiate endocytosis. In the cytoplasm, virus particles disassemble and the viral genome is released. With the help of both host and viral proteins, viral genome replication starts to make double-stranded RNA from the single-stranded positive-sense RNA [ssRNA(+)] genome, which is followed by transcription and replication to provide more mRNAs and new ssRNA(+) genomes. The RNA genome forms a nucleocapsid along with copies of the 12-kDa capsid protein. The nucleocapsid, in turn, is enveloped within a host-derived membrane modified with two viral glycoproteins to assemble into premature virus particles. Through processing in the Golgi complex, the mature virions are produced and released for the next round of infection.

Previous human and animal studies have established a causal relationship between ZIKV and microcephaly (6, 15–21). In the brain, neural stem cells, astrocytes, oligodendrocyte precursor cells, and microglia were found to be preferentially infected by ZIKV, whereas neurons were less susceptible to infection (22). In order to reach the fetal brain, viruses have to breach the placental barrier and transport from the maternal to the fetal circulatory system. In the placenta, the maternal blood and fetal blood are separated by placental barrier cells, including trophoblasts and endothelial cells. Recent studies have demonstrated that fetal endothelial cells or human umbilical vein endothelial cells (HUVEC) are permissive for ZIKV infection, making HUVEC a key cell model for ZIKV studies (23, 24).

In this study, we used HUVEC and astrocytes as the models to screen for candidate chemical compounds that can effectively inhibit ZIKV replication. By testing inhibitors and agonists of the 5'-AMP-activated protein kinase (AMPK), protein kinase A (PKA), and mitogen-activated protein kinase (MAPK) signaling pathways, we identified PKI 14-22 (PKI; a PKA inhibitor) and SB203580 (a p38 inhibitor) to be potent inhibitors that can suppress ZIKV replication with minimal cellular cytotoxicity. Further investigation showed that PKI significantly inhibited the replication of ZIKV from both the African and Asian/American clades at the postentry stage. Additionally, we showed that ZIKV infection of HUVEC did not induce PKA activation and PKI inhibited ZIKV replication by shutting down negative-sense RNA synthesis as well as viral protein expression. Our study demonstrates that PKI potentially suppresses ZIKV replication and, hence, is a potential candidate for therapeutic application.

RESULTS

Human umbilical vein endothelial cells are permissive for infection with ZIKV of both the African and Asian/American clades. In the placenta, fetal blood is separated from maternal blood by placental barrier cells, which are composed of trophoblasts and fetal endothelial cells. Recent studies have shown that both trophoblasts and fetal endothelial cells in the infected hosts are infected by ZIKV (6, 11, 16–18,

FIG 1 HUVEC are permissive for infection with ZIKV strains of both the African and Asian/American lineages. (A to D) Growth kinetics of ZIKV strains IbH 30656 (A), MR766 (B), H/FP/2013 (C), and PRVABC59 (D) in HUVEC. HUVEC were infected with ZIKV at an MOI of 1.0 for 1 h and then washed three times with PBS. The culture medium was replaced, and the supernatant was collected at 3, 24, 48, 72, and 96 h for plaque assay. (E to H) Kinetics of genome replication of ZIKV strains IbH 30656 (E), MR766 (F), H/FP/2013 (G), and PRVABC59 (H). ZIKV-infected HUVEC were lysed at the indicated time points. Both positive- and negative-sense viral genomes were measured by RT-qPCR. (I to L) Kinetics of viral protein synthesis of ZIKV strains IbH 30656 (I), MR766 (J), H/FP/2013 (K), and PRVABC59 (L). ZIKV-infected HUVEC were lysed at the indicated time points. Both nonstructural (NS1) and structural (capsid) viral proteins were measured by Western blotting.

23–25). To confirm that HUVEC were permissive for infection with all the ZIKV strains used in the study, we first evaluated the infectivity and replication of ZIKV in HUVEC. In this study, we used ZIKV strains from both the African and Asian/American clades, including IbH 30656 (African clade), MR766 (African clade), H/FP/2013 (French Polynesia, Asian clade), and PRVABC59 (Puerto Rico, American clade) (26). The pathologies observed in animal models based on the different virus strains are still unclear because each study has used a different ZIKV strain(s), resulting in different viral loads. In mouse models, strains from the African and Asian lineages were able to establish infection (27–30). Studies with pregnant animals focused on the strains recently isolated from the Americas or French Polynesia, which were able to cause development problems mimicking the microcephaly observed in humans (15, 20, 21, 31, 32). Additionally, ZIKV strains isolated from Brazil and injected into nonhuman primates at high dosages were able to cause alterations in the fetal brain (19). On the other hand, studies using other strains at much lower doses were unable to show the same effect (33, 34). The comparisons among the strains and their pathologies cannot be limited to the dosages used in different studies. At this point, there is no evidence that the strains from the Asian lineage are the only ones associated with the teratogenic effect in fetuses.

ZIKV was propagated in a mammalian cell system (Vero cells) to generate high titers of stocks. HUVEC were infected with various ZIKV strains at a multiplicity of infection (MOI) of 1.0, and virus production was continuously monitored (Fig. 1A to D). HUVEC were permissive for infection with all the ZIKV strains studied. High levels of ZIKV

progenies in the culture supernatants, determined by plaque assay, were detected at 72 and 96 h postinfection (hpi). We further examined the expression levels of intracellular viral genes and proteins in the ZIKV-infected HUVEC by reverse transcription-quantitative real-time PCR (RT-qPCR) and Western blotting, respectively. We found that all the strains reached the plateau at about 72 hpi, with strains IbH 30656, MR766, and PRVABC59 showing decreased expression levels of genes and proteins thereafter (Fig. 1E to L), which might have been a result of cell death associated with massive virus amplification and virion production. While the expression of positive- and negative-sense viral genomes showed similar trends in cells infected by strains IbH 30656, MR766, and PRVABC59, it was interesting that the negative-sense viral genome clearly lagged behind the positive-sense viral genome in cells infected by H/FP/2013 (Fig. 1E to H). Overall, the expression levels of intracellular viral genes and proteins were consistent with the levels of virus production observed in the culture supernatants of cells infected by the four ZIKV strains examined.

Screening for protein kinase inhibitors/agonists that can effectively inhibit ZIKV replication in HUVEC. Viruses often hijack host cellular signaling pathways to facilitate their infection and replication. Several pathways, such as the MAPK, PKA, and AMPK pathways, have been reported to play important roles in flavivirus replication (35–39). To determine if these pathways are essential for ZIKV infection, HUVEC were first preincubated with either an inhibitor or an agonist of a corresponding signaling pathway and then infected with ZIKV in the presence of the chemical compound. At 3 days postinfection (dpi), the amount of newly produced infectious virions in the culture supernatant was titrated. For screening purposes, we applied the chemicals at the concentrations commonly used in other applications, and at all concentrations tested the chemicals showed minimal cytotoxicity to HUVEC (Fig. 2A to G). Based on the results of the plaque formation assay, we found that ZIKV infection was effectively inhibited by the AMPK inhibitor compound C, the AMPK agonists metformin and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), the p38 inhibitor SB203580, and the PKA inhibitor PKI, while the extracellular signal-regulated kinase (ERK) inhibitor U0126 and Jun N-terminal protein kinase (JNK) inhibitor JNK inhibitor II had no effect (Fig. 2H). The AMPK inhibitor and agonists did not have any effect on host cell viability (Fig. 2A to C) but caused an approximately 60% to 80% decrease in virus production (Fig. 2H). Since both the activation and inhibition of AMPK activity resulted in a similar inhibitory effect, we concluded that the effect was not specifically related to AMPK's function; rather, it might have been due to an intracellular metabolic disorder and stress (40, 41). Due to the cytotoxicity of U0126 and JNK inhibitor II on HUVEC, they were used at relatively low concentrations, which did not cause any significant inhibition of ZIKV replication (Fig. 2D, E, and H). From the screening, we found that only the p38 inhibitor SB203580 and the PKA inhibitor PKI inhibited over 95% of ZIKV virion production without any visible toxicity on the host cells, indicating that both chemicals may be potential candidates for further development into therapeutic agents with activity against ZIKV (Fig. 2F to H). In this study, we focused on PKI and investigated its effect on ZIKV replication in depth.

PKI 14-22 inhibits ZIKV replication with an IC_{50} much higher than that toward PKA. PKI is a specific inhibitor of PKA (42). N-terminal myristoylation of this heat-stable peptide increases its cell membrane permeation. To further confirm the inhibitory effect of PKI, we first evaluated the effect of various doses of PKI on ZIKV. Following 1 h of preincubation of HUVEC with increasing concentrations of PKI, the cells were infected with ZIKV for an additional hour in the presence of PKI. After three intensive washes to remove unbound virions, we replaced the growth medium with fresh medium containing PKI and incubated the culture for three more days. Then, the culture supernatant was collected for plaque formation assay. As expected, PKI inhibition of ZIKV replication was dose dependent (Fig. 3A to D). PKI at a concentration of 10 μ M or lower did not show significant suppression, while at 20 or 40 μ M, it almost completely inhibited the replication of ZIKV strains from both the African and Asian/American clades (Fig. 3A to D). The half-maximal (50%) inhibitory concentrations (IC_{50})

FIG 2 Screening of protein kinase inhibitors/agonists that can inhibit ZIKV replication. (A to G) Effects of protein kinase inhibitors or agonists on the viability of HUVEC after 72 h of treatment. Cell viability was determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, and the results are expressed as the mean $\pm$ SD ($n = 3$) percentage of live cells. (H) Effects of protein kinase inhibitors or agonists on new progeny virion production. HUVEC preincubated with the indicated inhibitor or agonist were infected with ZIKV for 1 h. After three washes with PBS, culture medium containing the inhibitor or agonist was added. Three days later, the culture supernatant was collected for plaque assay. The effect of the inhibitor or agonist on ZIKV replication is expressed as a percentage of the viability for the nontreated group, and the results are presented as means $\pm$ SDs ($n = 3$). NS, not significant; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

of PKI for the IbH 30656, MR766, H/FP/2013, and PRVABC59 strains were 17.75 μM , 22.29 μM , 34.09 μM , and 19.19 μM , respectively. Hence, the IC_{50} was about 20 μM for all strains except the French Polynesia strain, for which the IC_{50} was slightly higher. These results indicate that PKI is able to broadly inhibit ZIKV replication regardless of the strain. Nevertheless, it needs to be emphasized that the PKI concentration required to effectively inhibit ZIKV replication is much higher than the concentration of 36 nM

FIG 3 PKI 14-22 inhibits ZIKV replication with an IC_{50} much higher than that toward PKA. (A to D) PKI inhibits virion production by ZIKV strains IbH 30656 (A), MR766 (B), H/FP/2013 (C), and PRVABC59 (D). HUVEC preincubated with PKI were infected with ZIKV for 1 h. After three washes with PBS, culture medium containing PKI was added. Three days later, the culture supernatant was collected for plaque assay.

of its nonmyristoylated version toward PKA, suggesting that PKI may affect ZIKV replication by inhibiting another target(s), in addition to the PKA pathway.

PKI 14-22 inhibits ZIKV replication in HUVEC at the postentry stage. Flavivirus infection is a complex event consisting of multiple steps, including virus attachment and entry, virus disassembly, viral protein expression, genome replication, as well as virion assembly and release from host cells (13). Hence, we determined the step of ZIKV infection that might be inhibited by PKI. First, we investigated whether PKI affected virus entry. Following 1 h of preincubation with PKI, HUVEC were infected with ZIKV for 2 h and extensively washed to remove unbound viruses. Then, the infected cells were trypsinized for 5 min to remove membrane-bound virions and lysed for RNA extraction. By analyzing the copy number of the positive-sense viral genome using RT-qPCR, we found that PKI did not significantly change the population of internalized virions for any of the ZIKV strains analyzed (Fig. 4A to D), indicating that PKI does not affect ZIKV entry.

Following virus internalization, virus-endosome membrane fusion, and release of the viral genome into the cytoplasm, ZIKV hijacks the host cell machinery to translate viral proteins and initiate viral genome replication. To investigate whether PKI affected the translation of viral proteins, we examined the expression levels of viral proteins by Western blotting. Treatment with PKI at 20 μ M and 40 μ M strongly inhibited the expression of both structural and nonstructural proteins (Fig. 4E to H). For the IbH 30656 and PRVABC59 strains, 10 μ M PKI reduced viral protein expression by approximately 50%, while PKI at this concentration produced no obvious change in viral protein expression for the MR766 and H/FP/2013 strains. Nonstructural proteins play essential roles in the synthesis of both negative- and positive-sense viral genomes. Thus, we tested the effect of PKI on viral genome replication by RT-qPCR. As expected, the synthesis of both negative- and positive-sense viral RNAs was significantly suppressed when viral protein levels were highly reduced by PKI (Fig. 4I to L). When these results are taken together, it can be seen that PKI inhibits ZIKV replication at the postentry stage by repressing the expression of viral proteins and the synthesis of viral genomes.

PKI 14-22 inhibits ZIKV replication by inhibiting the PKA pathway and another unknown target(s). A previous study has demonstrated that infection by hepatitis C virus (HCV), another flavivirus, increases the cAMP level and PKA activity to promote its infection (43). Despite the high PKI concentration required for inhibiting ZIKV replica-

FIG 4 PKI 14-22 inhibits the replication of ZIKV strains of both the African and Asian/American lineages at the postentry stage. (A to D) PKI does not affect the entry of ZIKV strains IbH 30656 (A), MR766 (B), H/FP/2013 (C), and PRVABC59 (D). HUVEC preincubated with PKI were infected with ZIKV for 2 h. Following intensive washes and 5 min of treatment with trypsin, the cells were lysed and the copy number of positive-strand viral RNA was measured by RT-qPCR. (E to H) PKI inhibits the viral protein expression of ZIKV strains IbH 30656 (E), MR766 (F), H/FP/2013 (G), and PRVABC59 (H) in a dose-dependent manner. Three days following ZIKV infection in the presence of PKI, the cells were lysed and viral protein levels were analyzed by Western blotting. (I to L) PKI inhibits the synthesis of both positive- and negative-sense RNAs of ZIKV strains IbH 30656 (I), MR766 (J), H/FP/2013 (K), and PRVABC59 (L) in a dose-dependent manner. Three days following ZIKV infection in the presence of PKI, the cells were lysed and the viral RNA level was analyzed by RT-qPCR.

tion, it remains possible that PKI may inhibit ZIKV replication through the PKA pathway. Activation of the PKA pathway is a multistep process which requires the phosphorylation of PKA at residue T197, converting the enzyme from an inactive state to an active state (57), as well as an increase in the intracellular cAMP level, inducing a conformational change in the regulatory subunits of PKA, which causes the subunits to unleash the two catalytic (activated) subunits (44). cAMP-responsive element-binding protein 1 (CREB1) is a direct downstream target of PKA, and activation of PKA results in CREB1 phosphorylation at residue S133 (45). CREB1 is an important transcription factor which binds to cAMP-responsive elements (CRE), thereby regulating the transcription of the downstream genes (44). A PKA agonist, forskolin (FSK), can activate the PKA pathway by stimulating adenylyl cyclase and raising the intracellular cAMP level (44). To determine if ZIKV infection could activate the PKA pathway, we first examined PKA phosphorylation at T197. Under our experimental conditions, we detected a high basal level of PKA phosphorylation at T197, which was not significantly altered following ZIKV infection (Fig. 5A). Treatment with FSK did not alter the level of PKA phosphorylation. PKI is a competitive inhibitor which mimics the protein substrate by binding to the catalytic site. As expected, PKI did not alter the PKA phosphorylation level. Next, we examined CREB1 activation. ZIKV infection did not alter CREB1 phosphorylation at S133 (Fig. 5A). Treatment with PKI reduced the level of CREB1 phosphorylation at S133 with or without ZIKV infection. As expected, treatment with FSK activated CREB1; however, it was not blocked by PKI, which was possibly due to the high concentration of FSK used (Fig. 5A).

The results presented above indicate that ZIKV infection does not activate the PKA pathway. However, it remains possible that the high endogenous level of the activated

FIG 5 ZIKV infection does not affect PKA activity but is enhanced by PKA activation. (A) Kinetics over time of CREB1 phosphorylation at S133 (p-CREB1 S133) and PKA phosphorylation at T197 (p-PKA T197) following ZIKV infection with or without FSK and/or PKI treatments. HUVEC were pretreated with FSK and/or PKI for 1 h, followed by mock or ZIKV infection at an MOI of 1.0 for 15 min and 30 min. Whole-cell lysates were collected for Western blot analysis for phospho-CREB1 (S133) and phospho-PKA (T197). Total CREB1 and PKA proteins were also detected. An anti- β -tubulin antibody was used to normalize the sample loading. (B) FSK promotes viral protein expression in HUVEC and cannot rescue the virus from the effect of PKI on viral protein expression. Cells were preincubated with FSK and/or PKI for 1 h, followed by ZIKV infection (MOI, 1.0) for an additional hour. After three washes with PBS, culture medium containing FSK and/or PKI was added. At the indicated time points, cells were lysed and viral protein levels were analyzed by Western blotting. The first column provides the results for noninfected HUVEC, while the second column provides the results for mock-infected cells. (C) FSK enhances the synthesis of both positive- and negative-sense ZIKV RNAs. Three days following ZIKV infection in the presence of FSK, the cells were lysed and the viral RNA level was analyzed by RT-qPCR. (D) FSK does not affect ZIKV virion production in HUVEC, nor can it rescue the virus from the effect of PKI. At the indicated time points, the culture supernatant was collected for quantification of the virus titer by plaque assay.

PKA pathway is required for ZIKV replication and the effect of PKI is through inhibition of the PKA pathway. If so, activation of the PKA pathway with FSK should further enhance ZIKV replication. Indeed, treatment with FSK increased the expression of ZIKV proteins (Fig. 5B) as well as the levels of both negative- and positive-sense ZIKV RNAs (Fig. 5C). Interestingly, the FSK-induced expression of ZIKV proteins was effectively blocked by PKI (Fig. 5B), albeit PKI failed to block the FSK-induced activation of CREB1 (Fig. 5A). Hence, the effect of these two chemicals may not be through the same target. Furthermore, treatment with FSK did not enhance the production of infectious virions, even though it significantly increased the expression of ZIKV proteins (Fig. 5D). PKI effectively inhibited the production of infectious virions with or without the presence of FSK (Fig. 5D).

The results so far indicate that ZIKV infection of HUVEC does not induce additional activation of PKA activity under our experimental conditions. The endogenous level of activated PKA was required for the efficient expression of ZIKV proteins and negative-

FIG 6 Astrocytes are permissive for ZIKV infection. (A) Growth kinetics of ZIKV strain IbH 30656 in astrocytes. Astrocytes were infected with ZIKV at an MOI of 1.0 for 1 h and then washed three times with PBS. The culture medium was replaced, and the supernatant was collected at 3, 24, 48, and 72 h for plaque assay. (B) Kinetics of genome replication of ZIKV strain IbH 30656. ZIKV-infected astrocytes were lysed at the indicated time points. The levels of both positive- and negative-sense viral genomes were measured by RT-qPCR. (C) Kinetics of viral protein synthesis of ZIKV strain IbH 30656. ZIKV-infected astrocytes were lysed at the indicated time points. Both nonstructural (NS1) and structural (capsid) viral proteins were measured by Western blotting.

and positive-sense ZIKV RNAs. Hence, inhibition of ZIKV replication could occur, at least in part, through the PKA pathway. However, PKI may also have an additional target that is likely involved in the late stage(s) of ZIKV replication. Our results suggest that the regulation of ZIKV by PKA may be different from that of other flaviviruses.

PKI 14-22 inhibits ZIKV replication in astrocytes. Previous studies showed that ZIKV preferentially infects neural stem cells, astrocytes, and microglia in the brain, whereas neurons were less susceptible to infection (22). To demonstrate that the inhibitory effect of PKI was not cell type specific, we tested whether PKI could repress ZIKV replication in human primary astrocytes. Consistent with the findings of a previous study (22), astrocytes were permissive for ZIKV infection (IbH 30656 strain) (Fig. 6). We detected viral protein expression, genome replication, and virion production at 3 dpi (Fig. 6). PKI at 20 and 40 μ M significantly reduced the production of progeny virions in astrocytes without any cytotoxicity (Fig. 7A and B). Accordingly, viral genome replication and protein expression were significantly inhibited (Fig. 7C and D), indicating that PKI may have antiviral activity in a broad range of cell types.

PKI 14-22 can inhibit viral replication after ZIKV infection occurs. We have shown that preincubation of HUVEC with PKI significantly inhibits viral replication and

FIG 7 PKI 14-22 inhibits the replication of ZIKV strain IbH 30656 in astrocytes. (A) PKI inhibits ZIKV virion production in astrocytes. Astrocytes preincubated with PKI were infected with ZIKV for 1 h. After three washes with PBS, culture medium containing PKI was added. Three days later, the culture supernatant was collected for plaque assay. (B) PKI does not affect the entry of ZIKV into astrocytes. Astrocytes preincubated with PKI were infected with ZIKV for 2 h. Following intensive washes and 5 min of treatment with trypsin, the cells were lysed and the copy number of positive-strand viral RNA was measured by RT-qPCR. (C) PKI inhibits the synthesis of both positive- and negative-sense RNAs of ZIKV in a dose-dependent manner. Three days following ZIKV infection in the presence of PKI, the cells were lysed and the viral RNA level was analyzed by RT-qPCR. (D) PKI inhibits the viral protein expression of ZIKV in a dose-dependent manner. Three days following ZIKV infection in the presence of PKI, the cells were lysed and viral protein levels were analyzed by Western blotting.

virion production. Next, we investigated whether PKI is able to inhibit ZIKV replication after infection has occurred in order to further dissect the stage of viral replication affected by the inhibitor. At various time points before or after ZIKV infection, we treated the cells with 40 μ M PKI and collected the culture supernatant at 3 dpi to evaluate the virus titer. We found that treatment of HUVEC with PKI at 24 hpi or earlier led to at least 85% or higher inhibition of virion production (Fig. 8A). Treatment within the first 8 h of ZIKV infection had the greatest inhibitory effect, which was comparable to that obtained with preincubation of the cells with PKI, while treatment at 48 hpi had

FIG 8 PKI 14-22 inhibits ZIKV replication at postinfection stages. (A to C) Before or after ZIKV infection, 40 μ M PKI was added to the culture medium at the indicated time points. (A) At 3 days after ZIKV infection, the culture supernatant containing newly produced virions was analyzed by plaque assay. (B and C) The synthesis of viral positive- and negative-sense RNAs was analyzed by RT-qPCR (B), and viral protein expression was evaluated by Western blotting (C). NT, no treatment.

only a marginal effect. We then checked the expression levels of viral proteins. We found that treatment with PKI at 24 hpi or earlier almost completely abolished the expression of both a structural protein (capsid) and a nonstructural protein (NS1) (Fig. 8B). RT-qPCR results showed that PKI, when added within the first 24 hpi, significantly decreased the levels of both negative- and positive-sense ZIKV RNAs (Fig. 8C). Taken together, these findings show that PKI suppresses ZIKV replication both before and after ZIKV infection is established. However, the PKI inhibitory effect is compromised if virus replication proceeds toward the late stage, when large amounts of the viral genome and proteins have accumulated in the infected cells.

PKI 14-22 inhibits ZIKV replication by suppressing negative-sense viral RNA synthesis. To further identify the step of ZIKV replication that is inhibited by PKI, HUVEC were infected with ZIKV for 24 h and then treated with PKI at 10, 20, or 40 μ M. Since all the viral proteins as well as both positive- and negative-sense viral RNAs were accumulated to a certain level at 24 hpi, we added the PKA inhibitor at this time point in an effort to identify the specific step of the viral replication cycle that is immediately suppressed by the inhibitor. At 4, 12, and 24 h following PKI addition, which corresponded to 28, 36, and 48 hpi, respectively, both viral RNA and protein samples were collected for analysis. We found that there was no significant difference between the PKI-treated and nontreated groups at 4 h and 12 h after addition of PKI for positive-sense viral RNA synthesis, and dramatic inhibition was observed only after 24 h of PKI treatment (Fig. 9A). In contrast, the level of negative-sense viral RNA showed a dose-dependent decrease as early as 4 h after PKI addition (Fig. 9B), indicating that the synthesis of negative-sense viral RNA is more sensitive to PKI treatment. By comparing the inhibition efficiency of PKI toward viral RNA synthesis, we found that treatment with 40 μ M PKI for 4 h resulted in approximately 70% inhibition of negative-sense viral RNA synthesis (Fig. 9C). The inhibition of negative-sense viral RNA synthesis occurred much earlier than that of positive-sense viral RNA synthesis (Fig. 9C), confirming that PKI mainly affects negative-sense RNA synthesis. Since the negative-sense RNA of flaviviruses often requires NS5 RNA-dependent RNA polymerase (RdRp) activity (46), further investigation can focus on whether and how PKI suppresses the ZIKV NS5 RdRp function.

We further examined the kinetics of the effect of PKI on viral protein expression. Without PKI treatment, the expression levels of viral proteins increased over time. When PKI was applied, we observed a significant decrease in the amount of viral proteins as early as 4 h following treatment (Fig. 9D). Such inhibition was more dramatic at later time points, indicating that PKI may also affect the translation of viral proteins.

PKI 14-22 does not interfere with host translation machinery to inhibit ZIKV replication. By screening genes essential for ZIKV replication using the clustered regularly interspaced short palindromic repeat (CRISPR) technology, previous studies have identified several host genes that play vital roles in regulating ZIKV replication (47, 48). Most of these genes are endoplasmic reticulum (ER) associated and functionally related to protein translation and modification. These functions include ER translocation, *N*-oligosaccharyltransferase (OST) activity, ER-associated protein degradation (ERAD), and so on. PKA is a multifunctional protein kinase that can regulate the expression of various downstream genes. Since PKI was found to block ZIKV replication in part by inhibiting PKA, we hypothesized that PKI might suppress ZIKV replication through inhibiting the expression of these critical genes. To test our hypothesis, HUVEC were infected with ZIKV in the presence of PKI, and then RT-qPCR was performed to evaluate the expression levels of these genes. No obvious change in the pattern of expression was observed for any of the genes tested (Fig. 10). The expression levels of most genes remained constant throughout ZIKV infection, with only STT3A showing a slight PKI dose-dependent increase in expression, suggesting that PKI may inhibit the host translation machinery and ZIKV protein expression through other downstream targets.

DISCUSSION

The reemergence of ZIKV across Pacific Islands and the Americas in recent years prompted emergent responses to this eminent health problem, especially in Latin

FIG 9 PKI 14-22 suppresses ZIKV replication by inhibiting viral protein translation and the synthesis of negative-sense viral RNA. (A and B) PKI at 10 μ M, 20 μ M, and 40 μ M was added to the culture medium at 24 hpi. Then, at 0, 4, 12, and 24 h after the addition of PKI, viral positive-sense RNA (A) and negative-sense RNA (B) levels were analyzed by RT-qPCR. (C) Efficiency of 40 μ M PKI inhibition of viral positive- and negative-sense RNAs calculated on the basis of the results presented in panels A and B. (D) Inhibition of ZIKV proteins by PKI added at different time points after ZIKV infection. At 24 hpi, PKI at 10 μ M, 20 μ M, and 40 μ M was added to the culture medium. Then, at 0, 4, 12, and 24 h after the addition of PKI, the viral protein expression level was analyzed by Western blotting.

American countries. The main pathological outcomes of ZIKV infection are neurological disorders, which include Guillain-Barré syndrome, mostly noted in the French Polynesia outbreak in 2013 (49, 50), and the increased numbers of microcephaly cases detected among newborns during the outbreak in Brazil in 2015 (7–10).

In the present study, we screened inhibitors of several host pathways in an effort to identify those that could block ZIKV infection. The regulation of MAPK pathways by

FIG 10 PKI 14-22 treatment does not affect the host cell protein translation machinery during ZIKV infection. (A to F) HUVEC pretreated with various concentrations of PKI were infected with ZIKV at an MOI of 1.0 for 1 h and then washed three times with PBS. The culture medium was replaced, and the cells were lysed at the indicated times. The expression levels of host genes related to protein translation, including SEC63 (A), SEC61B (B), STT3A (C), SPCS1 (D), SPCS3 (E), and SSR3 (F), were measured by RT-qPCR.

flaviviruses has been extensively investigated. HCV infection activates the Ras/Raf/MEK pathway, resulting in the attenuation of the interferon-JAK-STAT pathway and stimulation of HCV replication (51). Dengue virus serotype 2 (DENV-2) participates in liver inflammation by inducing the expression of various chemokines through activating all three MAPK pathways (52). YFV infection induces ERK1/2 activation, and treatment with U0126 reduces the level of YFV replication by 99% (38). A similar inhibitory effect of the ERK inhibitor was also observed in infections with dengue virus (DENV-2 and -3) and St. Louis encephalitis virus (SLEV) (38). AMPK, an intracellular energy sensor, plays a pivotal role in cellular metabolism, particularly in lipid and glucose metabolism (53). AMPK has general antiviral activity against multiple arboviruses from various families, including the flavivirus Kunjin virus, the togavirus Sindbis virus, and the rhabdovirus vesicular stomatitis virus (54). PKA activity is also important for flavivirus infection. HCV infection induces PKA activation in order to promote virus entry and infectivity (43). We chose these three signaling pathways because they are generally modulated during virus infection, and their activities play important roles in virus infection and replication.

We identified the PKA inhibitor PKI to be an inhibitor of ZIKV replication. Although ZIKV infection did not further alter the PKA activation status, treatment with the PKA agonist FSK enhanced the expression of ZIKV proteins and the viral genome, indicating that the endogenous activated PKA may regulate ZIKV replication. However, the PKI concentrations required for efficient inhibition of ZIKV replication were much higher than its 50% effective concentration for PKA inhibition. Furthermore, FSK did not increase the production of infectious virions. All of these findings indicate that PKI may block ZIKV replication by inhibiting another unknown target(s), in addition to the PKA pathway. We also tested the effects of several other PKA inhibitors, including PKI 6-22, PKI 5-24, and Rp-cAMP on ZIKV replication; however, none of them showed an inhibitory effect comparable to that of PKI (data not shown), confirming that PKI may suppress ZIKV replication through another unidentified cellular target(s).

Since there has not been a report of neurological diseases in humans as a result of ZIKV infection in Africa in its 60 years of circulation, questions regarding the pathogenicity of different viral strains have been raised. Both *in vitro* and animal studies have analyzed the differences in the pathologies caused by various ZIKV strains. MR766 is a

murine neurologically adapted strain because it has been passaged in suckling mouse brains for many years since its isolation in 1947. Rates of infection with this virus strain are higher than those for the recently isolated strains. In these studies, the viruses were generated in diverse cell lines, such as C6/36 or Vero cells, and were used at different passages and injected into the animals in different sites, with viral titers varying up to 108-fold. Hence, it is difficult to compare the virulence of different ZIKV strains on the basis of these results. There is no evidence to suggest that the Asian strains, including the ones most recently isolated in the Americas, are the only strains associated with microcephaly. For this reason, we examined four different strains of ZIKV, including IbH 30656 and MR766 from the African clade and H/FP/2013 and PRVABC59 from the Asian/American clade, in our current study.

Our results show that PKI is effective in suppressing the replication of ZIKV regardless of its origin. Although ZIKV strains have genetic compositions that may be associated with their different pathogenicities, PKI is able to overcome the genetic complexity and broadly inhibit ZIKV replication. However, further investigation is required to determine if this broadly inhibitory effect can prevent pathogenesis *in vivo*.

We have shown that PKI mainly targets the synthesis of negative-sense RNA and the expression of viral proteins. A previous study demonstrated that the synthesis of negative-sense RNA primarily depends on NS5 RdRp activity, whereas the synthesis of positive-sense RNA requires multiple additional enzymatic functions from both NS3 (helicase, ATPase, RNA triphosphatase) and NS5 (guanylyltransferase, methyltransferase) (46). Although there is no evidence so far showing that PKA directly phosphorylates NS5, it has been shown that NS5 could indeed be phosphorylated at Thr 449 in the RdRp fingers domain by protein kinase G, leading to the inactivation of its function (55). The role of phosphorylation of the RdRp Thr 449 residue is currently unclear. Mutation of this residue to histidine or glutamic acid in a DENV replicon abolished viral replication, indicating that Thr 449 is critical for viral RNA replication. It would be interesting to further explore the relationship between PKA or other PKI targets and NS5 posttranslational modification.

ZIKV protein translation and modification heavily rely on the host cell machinery. Recent CRISPR-Cas9-based screenings have confirmed the importance of these groups of host proteins, most of which are directly associated with the ER function (47, 48). Among these genes, SPCS1 and SPCS3 are signal peptidases which convert secretory and some membrane proteins to their mature forms by cleaving their signal peptides from their N termini; SEC61B and SEC63 are part of the SEC61 complex, which mediates protein translocation across the ER; STT3A is the catalytic subunit of the *N*-oligosaccharyltransferase (OST) complex, which catalyzes *N*-linked glycosylation; and SSR3 is a glycosylated ER membrane receptor associated with protein translocation across the ER membrane. Our results show that the expression levels of these genes in host cells do not change significantly following PKI treatment, suggesting that they are unlikely to be the downstream targets of PKA. Additional investigations are required to identify the PKI-regulated genes involved in ZIKV replication.

MATERIALS AND METHODS

Antibodies and reagents. Primary antibodies against PKA catalytic subunit (PKAc), phospho-PKAc (T197), phospho-CREB1 (S133), and CREB1 were purchased from Cell Signaling Technology (Danvers, MA). Primary antibody against the ZIKV capsid was obtained from GeneTex, Inc. (Irvine, CA), while primary antibodies against the ZIKV envelope (NDTGHETDENRAK) and NS1 (EAWRDYKYHPDSPRR) proteins were synthesized by Chempeptide Limited (Shanghai, China). Antibody to β -tubulin was purchased from Sigma-Aldrich (St. Louis, MO). Horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin and goat anti-rabbit immunoglobulin antibodies and Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin G (IgG) were obtained from Santa Cruz Biotechnology (Dallas, TX).

Kinase inhibitors and antagonists were obtained from the following sources: myristoylated protein kinase inhibitor (14–22) amide (PKI), compound C, U0126, SB203580, JNK inhibitor II, and forskolin (FSK) were from VWR International, LLC (Radnor, PA), and AICAR and metformin were from Cayman Chemical (Ann Arbor, MI).

Cell culture. Primary HUVEC were cultured in Vasculife vascular endothelial growth factor complete medium (Lifeline Cell Technology, Frederick, MD). African green monkey kidney Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and

1× penicillin-streptomycin (P/S) solution (Genesee Scientific, San Diego, CA). C6/36 *Aedes albopictus* cells were cultured in RPMI 1640 with 10% FBS and 1× P/S. HUVEC and Vero cells were cultivated at 37°C with 5% CO₂, and C6/36 *Aedes albopictus* cells were cultivated at 28°C with 5% CO₂. Simian virus 40-transformed astrocytes (CRL-8621), purchased from the American Type Culture Collection (Manassas, VA), were cultured in DMEM supplemented with 2% FBS and 1× P/S solution.

Virus preparation and titration. ZIKV stocks were propagated in Vero cells or C6/36 *Aedes albopictus* cells. Cells were inoculated with ZIKV at an MOI of 0.01 or 0.1, and the supernatants were harvested at between 48 and 96 hpi, depending on the ZIKV strain. The titers of the ZIKV stocks were determined by plaque assay on Vero cells. Briefly, a series of 10-fold dilutions of the virus inoculum were spread onto monolayers of Vero cells at 37°C for 1 h to initiate virus attachment to the cells. A mix of nutrient solution with agar (Lonza) was then added. The cells were maintained at 37°C for 5 days before the plaque assay was performed. For counting of the plaques, the cells were incubated with 10% formaldehyde and 0.1% crystal violet in 20% ethanol. These experiments were repeated three times.

Virus infection. HUVEC were infected at an MOI of 1.0 for 1 h. The cells were then washed with 1× phosphate-buffered saline (PBS) to remove unbound viruses, and fresh medium was added. To determine the effects of the kinase inhibitor on ZIKV infection, HUVEC grown to confluence in 36-mm dishes were first treated with the kinase inhibitor (PKI) for 1 h at 37°C prior to ZIKV infection. The cells were then infected with ZIKV for 1 h in the presence of the inhibitor. The cells were then washed with 1× PBS to remove unbound viruses, and the medium was replaced.

Western blot analysis. Cells infected with ZIKV for the times indicated above and in the figures were washed once with ice-cold PBS, and total protein was extracted. Total protein preparations were separated in sodium dodecyl sulfate-polyacrylamide electrophoresis gels, transferred to nitrocellulose membranes, and detected with antibodies. Specific signals were revealed with chemiluminescent substrates and recorded using a UVP multispectral imaging system (UVP LLC, Upland, CA).

RT-qPCR. The expression levels of viral genes were analyzed by reverse transcription-quantitative real-time PCR (RT-qPCR) using previously described procedures (56). Briefly, total RNAs from ZIKV-infected HUVEC were prepared with the TRI reagent as recommended by the manufacturer (Sigma-Aldrich). The RNA was treated with RNase-free DNase (Thermo Fisher Scientific, Inc., Waltham, WA) and reverse transcribed to obtain the first-strand cDNA using a Maxima reverse transcriptase system (Thermo Fisher Scientific). For each sample, a control reaction without reverse transcriptase was conducted in parallel. Quantitative PCR was then performed with the cDNA using gene-specific PCR primers. α -Tubulin was used as the internal control. Each sample was assayed with three repeats.

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