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Gabriela Miranda Ayusso

Avaliação de Peptídeos Sintéticos na Inibição do Vírus Chikungunya

São José do Rio Preto

2023

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Tese apresentada como parte dos requisitos para obtenção do título de Doutora em Microbiologia, junto ao Programa de Pós-Graduação em Microbiologia, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de São José do Rio Preto.

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RESUMO

O vírus Chikungunya (CHIKV) apresenta distribuição mundial e é considerado um grave problema de saúde pública. A natureza debilitante e crônica associada à infecção por CHIKV e a ausência de uma terapia antiviral aprovada demonstram a necessidade de fármacos específicos que atuem no ciclo replicativo do vírus. A descoberta do potencial terapêutico de diversos peptídeos despertou o interesse da comunidade científica nessa classe de substâncias que apresenta ampla atividade biológica, destacando a antiviral. O objetivo do presente trabalho foi avaliar o potencial antiviral do peptídeo sintético $(p\text{-BthTX-I})_2K$ e do protótipo peptídico sintético AG-Hecate e seus análogos PSSct1905 e PSSct1910 contra a infecção por CHIKV. A citotoxicidade dos peptídeos foi determinada em células de fibroblastos renais de hamster recém-nascido (BHK-21) e/ou células epiteliais de hepatocarcinoma humano (Huh-7) pelo ensaio de MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. O efeito antiviral foi investigado por meio da mensuração da atividade da proteína NanoLuciferase, codificada pelo CHIKV-NLuc. Todos os peptídeos exibiram atividade inibitória sobre o ciclo de replicação do CHIKV. O AG-Hecate e seu análogo PSSct1905 foram os mais ativos, apresentando ação antiviral maior que 91%. O peptídeo AG-Hecate e seus análogos atuaram em diferentes etapas do ciclo replicativo do CHIKV em células BHK-21 e Huh-7. O análogo PSSct1905 demonstrou efeito protetivo nas células contra a infecção viral. A entrada do CHIKV em ambas as linhagens celulares também foi afetada pelos peptídeos PSSct1905 e PSSct1910, desencadeando inibição da adsorção e internalização viral. Por fim, o protótipo AG-Hecate e seus dois análogos apresentaram atividade antiviral nas etapas de pós-entrada do ciclo de replicação viral nas duas células avaliadas. O peptídeo $(p\text{-BthTX-I})_2K$, por sua vez, exibiu ação anti-CHIKV ao interferir nas etapas iniciais do ciclo de infecção viral. O mesmo impediu a entrada do CHIKV em células BHK-21 por meio da inibição das etapas de adsorção e internalização viral. Além disso, observou-se que o efeito inibitório do peptídeo $(p\text{-BthTX-I})_2K$ na entrada viral foi mais significativa na adsorção do que na internalização do CHIKV em células BHK-21. Portanto, esse estudo destacou o peptídeo $(p\text{-BthTX-I})_2K$ e o protótipo peptídico AG-Hecate e seus análogos PSSct1905 e PSSct1910 como promissores candidatos a fármaco contra a infecção por CHIKV. Ademais, considerando as propriedades antivirais desses peptídeos, sugere-se a utilização das estruturas dos mesmos como base para o planejamento de novos antivirais de amplo espectro.

Palavras-chave: Chikungunya. Peptídeos. Antiviral.

ABSTRACT

Chikungunya virus (CHIKV) has a worldwide distribution and is considered a serious public health problem. The debilitating and chronic nature associated with CHIKV infection and the absence of approved antiviral therapy demonstrate the need for specific drugs that act on the virus's replicative cycle. The discovery of the therapeutic potential of several peptides aroused the interest of the scientific community in this class of substances that has broad biological activity, highlighting the antiviral. The aim of the present work was to evaluate the antiviral potential of the synthetic peptide (p-BthTX-I)$_2$K and the synthetic prototype peptide GA-Hecate and its analogs PSSct1905 and PSSct1910 against CHIKV infection. The cytotoxicity of the peptides was determined in baby hamster kidney fibroblast cells (BHK-21) and/or human hepatocarcinoma epithelial cells (Huh-7) by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The antiviral effect was investigated by measuring the activity of the NanoLuciferase protein, encoded by CHIKV-NLuc. All peptides exhibited inhibitory activity on the CHIKV replication cycle. AG-Hecate and its analog PSSct1905 were the most active, showing an antiviral action greater than 91%. The peptide GA-Hecate and its analogs acted at different stages of the CHIKV replicative cycle in BHK-21 and Huh-7 cells. The analog PSSct1905 demonstrated protective effect on cells against viral infection. CHIKV entry into both cell lines was also affected by peptides PSSct1905 and PSSct1910, triggering inhibition of viral attachment and internalization. Finally, the prototype GA-Hecate and its two analogs showed antiviral activity on the post-entry stages of the viral replication cycle in the two evaluated cells. The peptide (p-BthTX-I)$_2$K, in turn, exhibited anti-CHIKV action by interfering with the early stages of the viral infection cycle. It prevented the entry of CHIKV into BHK-21 cells by inhibiting the viral attachment and internalization steps. Furthermore, it was observed that the inhibitory effect of the peptide (p BthTX-I)$_2$K on viral entry was more significant on attachment than on internalization of CHIKV in BHK-21 cells. Therefore, this study highlighted the peptide (p-BthTX-I)$_2$K and the prototype peptide GA-Hecate and its analogs PSSct1905 and PSSct1910 as promising drug candidates against CHIKV infection. Besides, considering the antiviral properties of these peptides, it is suggested to use their structures as basis for the design of new broad-spectrum antivirals.

Keywords: Chikungunya. Peptides. Antiviral.

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LISTA DE ABREVIATURAS E SIGLAS

5-IT: análogo de adenosina 5-iodotubercidina

AG: ácido gálico

AG-Hecate: peptídeo Hecate conjugado ao ácido gálico

AMPs: antimicrobial peptides

APD3: base de dados de peptídeos antimicrobianos

AS: asiático

BHK-21: células de fibroblastos renais de hamster recém-nascido

BthTX-I: Bothropstoxin-I

C: control

CAPES: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CC_{50}: 50% cytotoxic concentration

CHIKV: vírus Chikungunya

CHIKV-NLuc: CHIKV expressing *nanoluciferase* reporter

CMC: carboxymethylcellulose

CMV: human cytomegalovirus

CNPq: Conselho Nacional de Desenvolvimento Científico e Tecnológico

CPP: cell-penetrating peptide

C_t: cycle threshold

CZS: congenital Zika syndrome

DC-SIGN: lectina dependente de cálcio do tipo C

DMSO: dimethylsulfoxide

DENV: vírus da Dengue

DMEM: Dulbecco's Modified Eagle's Medium

EC_{50}: 50% effective concentration

FAPESP: Fundação de Amparo à Pesquisa do Estado de São Paulo

FBS: fetal bovine serum

FDA: Food and Drug Administration

GA: gallic acid

GAGs: glycosaminoglycans

GA-Hecate: peptide conjugated to the GA

gp41: glicoproteína 41

HCV: vírus da hepatite C

HIV-1: vírus da imunodeficiência humana do tipo 1

h.p.i.: hours post-infection

h.p.t.: hours post-transfection

HSF: células de fibroblastos de pele humana

HSV: vírus da herpes simples humano

Huh-7: células epiteliais de hepatocarcinoma humano

LCSA: leste/centro/sul-africano

MERS-CoV: coronavírus relacionado à síndrome respiratória do Oriente Médio

MNTC: maximum nontoxic concentration

MOI: multiplicity of infection

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MW: molecular weight

NLuc: NanoLuciferase

OE: oeste africano

OI: oceano índico

OMS: Organização Mundial de Saúde

ONNV: alfavírus o'nyong-nyong

ONU: Organização das Nações Unidas

OPTI-MEM: Reduced Serum Medium

ORF: open reading frame

P/S: penicillin/streptomycin

PAHO: Pan American Health Organization

PAMs: peptídeos antimicrobianos

PAVdb: base de dados de peptídeos antivirais validados experimentalmente

PAVs: peptídeos antivirais

PBS: phosphate-buffered saline

PFU/mL: unidades formadoras de placa por mL

PHB1: proteína de membrana proibitina do tipo 1

PtdSer: phosphatidylserine

PVEERs: phosphatidylserine (PtdSer)-mediated virus entry-enhancing receptors

qRT-PCR: reverse-transcription–quantitative RT–PCR

SARS-CoV: coronavírus relacionado à síndrome respiratória aguda grave

SD: standard deviation

SI: selectivity index

SPPS: solid phase peptide synthesis

TIM: mucina

UFU: Universidade Federal de Uberlândia

UNESP: Universidade Estadual Paulista Júlio de Mesquita Filho

VC: vehicle control

Vero: células renais de macaco verde africano

ZIKV: vírus Zika

SUMÁRIO

Capítulo I

1. INTRODUÇÃO

1.1. Arboviroses

As arboviroses são doenças causadas por um grupo de vírus (arbovírus) que tem os artrópodes hematófagos como vetores de transmissão (SIGFRID *et al.*, 2018; HIGUERA; RAMÍREZ, 2019; LUNA *et al.*, 2019). Essas doenças ocorrem de forma esporádica, endêmica ou epidêmica. As consequências sociais e econômicas das arboviroses epidêmicas que surgiram nos últimos 50 anos e suas contribuições na mortalidade mundial ressaltaram o grave problema de saúde pública que essas doenças representam (WILDER-SMITH *et al.*, 2017; BRAACK *et al.*, 2018; GIRARD *et al.*, 2020). A ocorrência e a extensão da disseminação geográfica de epidemias de arboviroses aumentou de modo acelerado nos últimos 30 anos, ocorrendo de maneira global nos trópicos atualmente (GIRARD *et al.*, 2020).

Atualmente, mais de 500 espécies de arbovírus pertencentes à 14 famílias diferentes foram relatadas, sendo mais de 100 espécies patógenos de humanos ou animais (KOLAWOLE *et al.*, 2018). Os arbovírus que causam doenças em humanos ou animais pertencem à quatro famílias principais: *Peribunyaviridae*, *Flaviviridae*, *Togaviridae* e *Sedoreoviridae* (KOLAWOLE *et al.*, 2018; SUKHRALIA *et al.*, 2019). Esses vírus predominam em regiões tropicais e subtropicais onde as condições são favoráveis para a reprodução dos vetores. Porém, esses patógenos também podem ser encontrados em regiões temperadas do mundo (HIGUERA; RAMÍREZ, 2019; SUKHRALIA *et al.*, 2019).

A transmissão e a disseminação das doenças arbovirais dependem do contato frequente entre patógeno (vírus), vetor (hospedeiro invertebrado) e hospedeiro vertebrado (BRAACK *et al.*, 2018). Os arbovírus apresentam uma ampla variedade de hospedeiros. Entre os vertebrados, destacam-se os mamíferos e as aves. Em relação aos invertebrados, os mosquitos e os carrapatos são os principais vetores. Os mosquitos do gênero *Aedes* e *Culex* são os principais mosquitos associados com a transmissão de arbovírus (SUKHRALIA *et al.*, 2019).

Os invertebrados infectados por arbovírus não demonstram sinais clínicos (KOLAWOLE *et al.*, 2018). Em contrapartida, as infecções arbovirais em vertebrados podem resultar em sintomas que variam desde uma síndrome febril benigna até encefalite, hemorragia, meningite e outros sintomas graves (LIANG *et al.*, 2015; KOLAWOLE *et al.*, 2018). Dessa forma, após a infecção por arbovírus, os hospedeiros silvestres raramente exibem morbidade e mortalidade enquanto essas condições são comumente observadas nos hospedeiros urbanos (GOULD *et al.*, 2017).

A transmissão dos arbovírus ocorre principalmente por transmissão horizontal entre os hospedeiros vertebrados e invertebrados. A competência de um vetor é definida como a capacidade de contrair e transmitir um determinado patógeno, sendo determinada por diversos fatores, tais como temperatura, ambiente, precipitação e saúde do invertebrado (CIOTA; KEYEL, 2019; SUKHRALIA *et al.*, 2019). Ciota e Keyel afirmam que a influência da temperatura na competência vetorial pode variar de acordo com a espécie do vetor e do vírus, sendo mais recorrente observar o aumento da competência do vetor com a elevação da temperatura. O vetor competente adquire o vírus pela ingestão de sangue virêmico do hospedeiro vertebrado. Nos casos de mosquitos, o vírus infecta e se replica nas células epiteliais do intestino médio, permeia a lâmina basal do intestino, adentra a hemocele, infecta e se replica nas glândulas salivares até formar uma quantidade suficiente de partículas virais na saliva (CIOTA; KEYEL, 2019). Dessa forma, esse vetor pode transmitir o patógeno para outro hospedeiro vertebrado susceptível e não infectado. Em casos mais raros, o arbovírus pode ser transmitido para o hospedeiro vertebrado sem a presença do vetor por meio de transmissão vertical ou sexual, transfusão sanguínea ou transplante de órgãos (CIOTA; KEYEL, 2019; HIGUERA; RAMÍREZ, 2019; SUKHRALIA *et al.*, 2019).

A mutação no genoma viral é um dos fatores que pode contribuir para a disseminação de doenças arbovirais. Pequenas modificações no material genético dos arbovírus podem influenciar na susceptibilidade e competência vetorial, promovendo aumento da eficiência de transmissão viral em vetores com baixa competência ou desencadeando o surgimento de novos vetores para disseminar o vírus (BRAACK *et al.*, 2018). A substituição do aminoácido alanina pela valina na posição 226 da glicoproteína E1 presente no envelope do vírus Chikungunya (CHIKV) resultou em aumento da susceptibilidade e competência do *A. albopictus*. A adaptação do CHIKV ao novo vetor possibilitou sua dispersão para regiões além da África, como Ásia e norte da Itália (BRAACK *et al.*, 2018; WEETMAN *et al.*, 2018).

Os vetores de arbovírus também podem sofrer mutações em seu genoma e seleções adaptativas que podem favorecer a propagação de arbovírus. Pequenas alterações no material genético dos mosquitos podem conferir resistência a diversos inseticidas, impossibilitando o controle desses vetores e consequentemente, viabilizando a manutenção das arboviroses. Nos últimos anos, verificou-se uma tendência crescente de resistência a inseticidas por *A. aegypti*, *A. albopictus*, *C. pipiens* e *C. quinquefasciatus*. A adaptação de diversos vetores ao ambiente urbano também é um fator que propiciou o alastramento das infecções arbovirais. O *A. aegypti* é exemplo de um mosquito da área silvestre que migrou e se adaptou ao meio urbano,

transmitindo diversos vírus com diferentes patologias para os seres humanos (BRAACK *et al.*, 2018).

Além da diversidade de espécies e da ampla distribuição dos vetores, outros fatores contribuem para a grande dispersão mundial das arboviroses. Segundo estimativas da Organização das Nações Unidas (ONU), haverá 9,6 bilhões de pessoas no mundo em 2050. O aumento da densidade populacional resulta na expansão dos ambientes urbanos, provocando a migração de espécies silvestres para áreas urbanas e consequentemente, alastramento de doenças arbovirais para o meio urbano (BRAACK *et al.*, 2018; LUNA *et al.*, 2019). Além disso, a urbanização crescente gera mudanças climáticas mundiais que propiciam um ambiente favorável para os vetores. De acordo com relatórios emitidos pelo Painel Intergovernamental de Mudanças Climáticas, está ocorrendo um aquecimento constante do planeta que irá persistir pelo restante desse século (BRAACK *et al.*, 2018). Outra consequência da urbanização é o fenômeno denominado "ilhas de calor", definido como um microclima com temperaturas mais altas comparado às áreas circundantes com vegetação. A temperatura dessas "ilhas de calor" beneficia a sobrevivência e o desenvolvimento de mosquitos que estão adaptados com temperaturas altas, como o *A. aegypti* (MORDECAI *et al.*, 2020). Por fim, o aumento populacional e a urbanização também resultam em condições ideais para a proliferação de vetores, tais como habitações inadequadas, desperdício de resíduos e acúmulo de lixo (GIRARD *et al.*, 2020).

A ausência de um controle efetivo dos vetores impossibilita a desaceleração da disseminação das arboviroses (GIRARD *et al.*, 2020). Quando os vetores são representados por mosquitos, as formas tradicionais de controle consistem em eliminação ou diminuição de hábitats larvais (métodos físicos, biológicos ou químicos) e populações adultas do mosquito (inseticidas químicos). A aplicação de inseticidas é a estratégia mais utilizada em casos de surto de emergência, sendo implementada em nível global. Porém, a resistência a inseticidas disseminou-se em diversas espécies de mosquitos, como *A. aegypti* e *A. albopictus*, prejudicando o refreamento da proliferação de doenças arbovirais (DUSFOUR *et al.*, 2019).

O aumento da população mundial juntamente com o processo de globalização desencadearam o aumento do movimento de pessoas por meio de viagens internacionais e a intensificação do comércio mundial (LIANG *et al.*, 2015; BRAACK *et al.*, 2018). No ano de 2016, o aumento do turismo na Ásia, Pacífico e África foi maior que 8%, sendo essas regiões as que abrigam maior quantidade de casos de arbovírus no mundo. A demanda mundial por produtos importados também aumentou de maneira significativa, principalmente nas regiões da Ásia, África e América Latina. Entre 1993 e 2006, o aumento do transporte marítimo no mundo

foi maior que 27% (BRAACK *et al.*, 2018). Em 2018, mais de três bilhões de indivíduos utilizaram o transporte aéreo. Essas condições propiciaram a rápida disseminação e introdução de vetores e consequentemente, arboviroses em novas áreas geográficas (LIANG *et al.*, 2015; BRAACK *et al.*, 2018; GIRARD *et al.*, 2020).

1.2. Vírus Chikungunya (CHIKV)

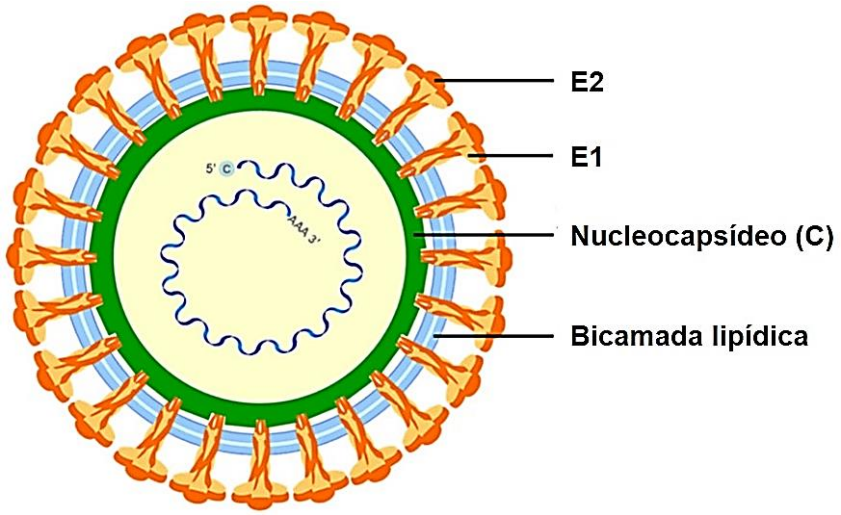
O CHIKV é um arbovírus que pertence à família *Togaviridae* e é agrupado dentro do gênero *Alphavirus*. Esse vírus foi descoberto em 1952 no atual sul da Tanzânia e os primeiros casos foram tratados como infecção por vírus da Dengue (DENV) (SILVA *et al.*, 2018; VAIRO *et al.*, 2018; MATUSALI *et al.*, 2019). O termo "Chikungunya" advém de uma palavra no idioma Kimakonde do sul da Tanzânia e significa "tornar-se contorcido", remetendo-se ao aspecto curvado de pessoas que apresentam artralgia, principal sintoma da infecção por CHIKV. Atualmente, o CHIKV apresenta distribuição mundial e é considerado um grave problema de saúde pública. De acordo com a Organização Mundial de Saúde (OMS), casos de CHIKV já foram identificados em mais de 110 países da Ásia, África, Europa e América. Desde 2005, foram relatados mais de 2 milhões de casos mundiais (WHO, 2022). No continente americano, mais de 270 mil casos foram descritos em 2022. Dentre os países da América, o Brasil é o que apresentou maior incidência, representando 96,9% dos casos de CHIKV (PAHO, 2023).

1.2.1. Caracterização e Ciclo de Replicação

O CHIKV é um vírus esférico e envelopado com um diâmetro de aproximadamente 70 nm. O vírion apresenta um genoma de RNA de polaridade positiva e de cadeia simples com tamanho aproximado de 11,5 kb, sendo poliadenilado na extremidade 3' e demonstrando uma capa de 7-metilguanosina na extremidade 5'. O genoma contém duas ORFs: (1) ORF 5' codifica uma poliproteína não-estrutural que é processada em quatro proteínas não estruturais (nsP1, nsP2, nsP3 e nsP4) que fazem parte do complexo de replicação viral; e (2) ORF 3' codifica uma poliproteína estrutural que é processada em seis proteínas estruturais principais (C-E3-E2-6K/TF-E1) (CUNHA; TRINTA, 2017; HIGUERA; RAMÍREZ, 2019; SUKHRALIA *et al.*, 2019). O material genético do CHIKV é envolto por um capsídeo com simetria icosaédrica, constituído por 240 cópias da proteína C (241 aminoácidos). A partícula do CHIKV apresenta um envelope constituído por uma bicamada lipídica derivada da membrana plasmática das células hospedeiras na qual estão ancoradas 80 fibras formadas por trímeros das glicoproteínas E1 e E2 (Figura 1) (CUNHA; TRINTA, 2017; SHARMA *et al.*, 2018).

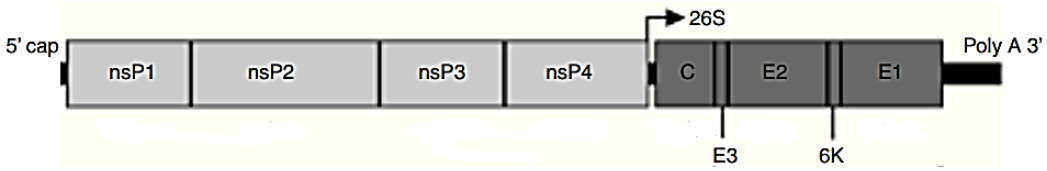
Figura 1. Representação esquemática (A) da partícula e (B) do genoma do CHIKV

A)



Fonte: Adaptado de (METZ; PIJLMAN, 2011)

B)



Fonte: Adaptado de (GALÁN-HUERTA *et al.*, 2015)

As glicoproteínas E1 (439 aminoácidos) e E2 (423 aminoácidos) tem sua função na entrada do CHIKV na célula hospedeira, portando os principais epítopos virais. A proteína E2 se liga ao receptor da célula, sendo o principal alvo dos anticorpos neutralizantes. A proteína E1, por sua vez, exibe um peptídeo de fusão hidrofóbico, apresentando papel crucial na fusão da membrana viral e celular. Os aminoácidos glicina e histidina nas posições 91 e 230, respectivamente, são responsáveis pela atuação da glicoproteína E1 na fusão das membranas viral e celular. As proteínas E1 e E2 formam heterodímeros sendo que a última encobre o peptídeo de fusão presente na primeira com o objetivo de evitar a fusão da membrana viral e celular de forma prematura. Os dímeros dessas proteínas transformam-se em trímeros e constituem estruturas denominadas spikes na superfície da partícula do CHIKV. A glicoproteína E1 trata-se de uma proteína de membrana do tipo II que demonstra três domínios barril beta (I, II e III). O peptídeo de fusão localiza-se na extremidade distal do domínio II da proteína E1. A glicoproteína E2, por sua vez, faz parte da superfamília das imunoglobulinas e exibe três domínios de imunoglobulina (A, B e C) (SUBUDHI *et al.*, 2018; SCHNIERLE, 2019).

A proteína E3 (64 aminoácidos) tem seu papel na translocação da poliproteína E3-E2-6K-E1 ou E3-E2-TF para o retículo endoplasmático para a formação do spike viral (TANABE *et al.*, 2018), além de evitar que o heterodímero E2-E1 se funda de maneira prematura com a membrana da célula hospedeira (SUBUDHI *et al.*, 2018). A proteína 6K (61 aminoácidos) promove o aumento da permeabilidade da célula para cátions monovalentes, desempenhando função no brotamento do vírion durante o processo de infecção. A proteína TF é a consequência da extensão da extremidade C-terminal da proteína 6K, apresentando a propriedade similar de canal seletivo para cátions e exibindo participação na montagem e liberação das partículas do CHIKV da célula hospedeira (TANABE *et al.*, 2018).

As proteínas não-estruturais do CHIKV são essenciais para que ocorra a replicação do vírus no citoplasma da célula hospedeira (BARR; VAIDHYANATHAN, 2019). A enzima nsP1 apresenta atividade de guanina-N7-metiltransferase e guanililtransferase, sendo responsável por adicionar um cap à extremidade 5’ do RNAm genômico e subgenômico. Esse capeamento, por sua vez, é crucial para que ocorra a tradução do RNAm viral, além de evitar a degradação do RNAm do CHIKV por 5’-exonucleases da célula hospedeira. A proteína nsP1 também permite, por meio da presença de uma hélice anfipática (entre os aminoácidos 245 e 264) e cisteínas palmitoiladas (417 a 419), que o complexo de replicação viral se ancore a microdomínios de membrana enriquecidos com colesterol, processo necessário para a replicação do CHIKV (KOVACIKOVA; VAN HEMERT, 2020).

A proteína nsP2 (798 aminoácidos) apresenta atividade de nucleosídeo trifosfatase, helicase, RNA 5’ trifosfatase, protease e RNA metiltransferase dependente de SAM. As atividades de nucleosídeo trifosfatase e helicase permitem o desenrolamento do RNA de fita dupla durante a replicação do CHIKV. A atividade RNA 5’ trifosfatase da enzima nsP2 é responsável pela remoção do γ-fosfato da extremidade 5’ do RNA antes da transferência da estrutura cap-0. A atividade de protease, por sua vez, faz com que a proteína nsP2 tenha papel no processamento das poliproteínas nsP123 e nsP1234. Por fim, a enzima nsP2 atua, por meio da atividade RNA metiltransferase dependente de SAM, na inibição da resposta do interferon. Essa proteína se desloca para o núcleo da célula hospedeira e induz a poliubiquitinação da subunidade catalítica da RNA polimerase II dependente de DNA, causando bloqueio do processo de transcrição do hospedeiro e efeitos citopáticos (KOVACIKOVA; VAN HEMERT, 2020).

A proteína nsP3 (530 aminoácidos) recruta proteínas de ligação ao RNA que desempenham papel na formação de pré-complexos de replicação do CHIKV, além de afetar o processo de tradução da célula hospedeira. Ademais, essa enzima apresenta duas atividades: (1)

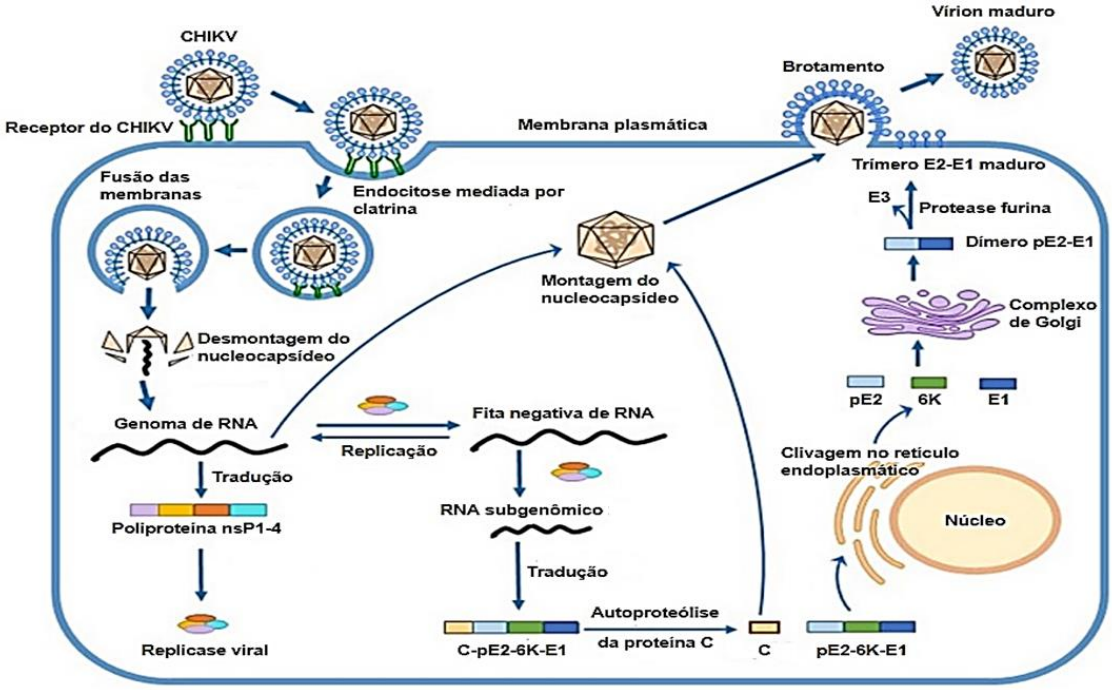
ligação à ADP-ribose em proteínas celulares, o que possibilita o início da síntese de proteínas não-estruturais do CHIKV e formação dos complexos de replicação viral; e (2) hidrólise de resíduos ribosilados de ADP em proteínas celulares, permitindo a expansão de complexos de replicação do CHIKV (KOVACIKOVA; VAN HEMERT, 2020).

A proteína nsP4 (611 aminoácidos) é uma RNA polimerase dependente de RNA que atua na síntese da fita negativa de RNA que irá servir como molde para a síntese do RNA genômico e subgenômico de fita positiva. Além disso, essa proteína demonstra atividade adenililtransferase que desencadeia a inserção de uma cauda poli-A na extremidade 3' do genoma do CHIKV (KOVACIKOVA; VAN HEMERT, 2020).

O ciclo de replicação do CHIKV (Figura 2) se inicia pela ligação da glicoproteína viral E2 com receptores específicos presentes nas células hospedeiras do vírus. Após essa interação, o CHIKV adentra a célula e atravessa o citoplasma por meio da endocitose mediada por clatrina. Com a acidificação do endossomo, ocorre alteração conformacional da glicoproteína viral E1, resultando na fusão das membranas viral e endossomal já que há exposição do peptídeo de fusão hidrofóbico da proteína E1. Após ocorrer esse processo, o nucleocapsídeo é liberado no citoplasma da célula, sendo, em seguida, desmontado para que ocorra a liberação do RNA viral (ABDELNABI *et al.*, 2015; SCHNIERLE, 2019).

O material genético do CHIKV é primeiramente traduzido pela maquinaria celular, produzindo a poliproteína não-estrutural (nsP1-4) que, por sua vez, é processada no precursor nsP123 e na proteína nsP4, formando um complexo inicial de replicação do vírus, denominado replicase viral. Este é responsável pela síntese da fita negativa de RNA que é utilizada como molde para a produção da fita positiva do RNA genômico e subgenômico. O RNA subgenômico serve como RNAm para a formação do polipeptídeo estrutural (C-pE2-6K-E1) que dá origem à proteína C, liberada por sua atividade de autoprotease, e ao polipeptídeo pE2-6K-E1, processado no retículo endoplasmático. As glicoproteínas pE2 e E1 formam heterodímeros que atravessam o complexo de Golgi. Ao se deslocar pela membrana celular, a proteína pE2 é clivada pela enzima furina e proteinases do tipo furina da célula hospedeira, resultando nas proteínas maduras E2 e E3. Por fim, os nucleocapsídeos são montados no citoplasma celular, obtendo um envelope de bicamada lipídica com glicoproteínas E1 e E2 por meio do brotamento na membrana plasmática da célula hospedeira (ABDELNABI *et al.*, 2015).

Figura 2. Representação esquemática do ciclo de replicação do CHIKV



Fonte: Adaptado de (ABDELNABI *et al.*, 2015)

1.2.2. Epidemiologia

Atualmente, existem quatro genótipos do CHIKV relacionados com a localização geográfica: leste/centro/sul-africano (LCSA), oeste africano (OE), asiático (AS) e oceano índico (OI). O último representa um grupo monofilético dentro da linhagem LCSA que se originou durante a disseminação do CHIKV no continente asiático. Estudos com sequências parciais do gene que codifica a glicoproteína E1 e com a sequência total do genoma viral demonstram as diferenças existentes no tamanho do material genético entre e dentro dessas linhagens virais geograficamente estabelecidas. A grande variação no genoma do CHIKV é atribuída principalmente à ineficiência da RNA polimerase dependente de RNA (nsP4) em reparar erros durante a biossíntese de RNA, originando uma mutação, no mínimo, em cada nova fita de RNA (HIGUERA; RAMÍREZ, 2019).

Entre os anos de 1952 e 2018, o CHIKV provocou mais de 70 epidemias mundiais (MATUSALI *et al.*, 2019). Após o primeiro surto na Tanzânia em 1952, infecções por CHIKV foram relatadas em outras regiões da África, como Uganda e Senegal. Uma mutação no gene que codifica a glicoproteína E1 (A226V) resultou em aumento da replicação viral no intestino médio e aumento da viremia em glândulas salivares de mosquitos da espécie *A. albopictus* (BRAACK *et al.*, 2018; HIGUERA; RAMÍREZ, 2019; MATUSALI *et al.*, 2019). O aumento

da susceptibilidade e competência desse mosquito ao CHIKV desencadeou a expansão geográfica desse arbovírus para outras áreas além da África, como Ásia, ilhas do Oceano Índico, Europa e América. Além disso, a vetorização do CHIKV por *A. albopictus* explica a disseminação do vírus para locais onde não há circulação do *A. aegypti* (HIGUERA; RAMÍREZ, 2019; MATUSALI *et al.*, 2019).

A partir de 1960, casos de infecções por CHIKV foram registrados em países do sudeste asiático, como Tailândia e Índia. Após a epidemia que ocorreu no Quênia em 2004, o vírus dispersou-se para as ilhas do Oceano Índico (BRAACK *et al.*, 2018; HIGUERA; RAMÍREZ, 2019; MATUSALI *et al.*, 2019). Em 2005, 266 mil pessoas foram infectadas na Ilha da Reunião (BRAACK *et al.*, 2018). A ausência do *A. aegypti* nessa ilha representou a primeira evidência de envolvimento de um outro vetor na transmissão do CHIKV (MATUSALI *et al.*, 2019). Os primeiros casos de infecção por CHIKV no continente europeu foram relatados no norte da Itália em 2007, disseminando-se posteriormente para outros países europeus, como França, Croácia e Espanha (HIGUERA; RAMÍREZ, 2019; MATUSALI *et al.*, 2019). Na América, os primeiros casos de CHIKV foram registrados na ilha caribenha de Saint-Martin em 2013 (BRAACK *et al.*, 2018; HIGUERA; RAMÍREZ, 2019; MATUSALI *et al.*, 2019). A presença em grande quantidade do *A. aegypti* e o elevado fluxo de turistas entre as ilhas do Caribe e os países da América resultaram em epidemias do CHIKV em 45 países americanos, incluindo o Brasil no ano de 2014 (HIGUERA; RAMÍREZ, 2019; MATUSALI *et al.*, 2019). O surto mais recente do vírus ocorreu no Sudão em 2018, com 13978 casos confirmados (MATUSALI *et al.*, 2019). De modo geral, as epidemias causadas por CHIKV ocorrem como surtos remotos intercalados com períodos de silêncio epidemiológico, sendo afetadas por diversos aspectos, como susceptibilidade da população ao arbovírus, interação do vetor com o vírus, competência vetorial e condições ambientais que interferem no ciclo biológico do mosquito (SILVA *et al.*, 2018; HIGUERA; RAMÍREZ, 2019).

1.2.3. Transmissão

Há dois tipos de ciclo de transmissão do CHIKV: o silvestre e o urbano. No ciclo de transmissão silvestre, o vírus é transmitido para primatas não-humanos principalmente por espécies fêmeas infectadas do gênero *Aedes*, como *A. africanus*, A. *furcifer*, *A. cordellieri*, *A. taylori*, *A. neoafricanus*, *A. luteocephalus*, *A. vittatus* e *A. fulgens* (BRAACK *et al.*, 2018; SILVA *et al.*, 2018; SUKHRALIA *et al.*, 2019) (TANABE et al., 2018). Além dos primatas não-humanos, alguns estudos indicam o envolvimento de morcegos e roedores como hospedeiros do CHIKV no ciclo silvestre (MATUSALI *et al.*, 2019). Esse ciclo ocorre

principalmente na África, sendo responsável pela manutenção do vírus durante os períodos interepidêmicos (MATUSALI *et al.*, 2019; SUKHRALIA *et al.*, 2019). No ciclo de transmissão urbano, *A. aegypti* e *A. albopictus* são os principais transmissores do CHIKV, sendo aquele também o principal vetor do vírus Zika (ZIKV) e do DENV (SILVA *et al.*, 2018; MATUSALI *et al.*, 2019; SUKHRALIA *et al.*, 2019). A infecção simultânea do *A. aegypti* pelos três vírus não compromete a competência do vetor, podendo ocorrer casos de cotransmissão (SILVA *et al.*, 2018).

O mosquito é infectado por CHIKV por meio da ingestão de sangue de um hospedeiro vertebrado virêmico que apresente títulos virais variando de 10^3 a 10^5 unidades formadoras de placa por mL (PFU/mL) (MATUSALI *et al.*, 2019). A subunidade β da ATP sintase é citada como provável receptor das células de mosquitos para interação com a glicoproteína E2 do CHIKV. Estudos evidenciaram que a inibição dessa proteína por anticorpos ou a regulação negativa da mesma por siRNA desencadeou um declínio da entrada e consequentemente, da formação de partículas virais nas células de mosquitos (SCHNIERLE, 2019). O vírus inicia sua replicação quando atinge o intestino médio do vetor ao penetrar no epitélio por endocitose mediada por clatrina (MATUSALI *et al.*, 2019). Dentro de aproximadamente três a dez dias, o CHIKV alcança as glândulas salivares do inseto, sofre replicação e se mantém nas células acinares sem provocar efeito citopático (SILVA *et al.*, 2018; MATUSALI *et al.*, 2019). Os vetores demonstram diferentes níveis de competência para diferentes isolados do CHIKV. Estudos sugerem que a proteína viral nsP3 esteja envolvida nessa especificidade vetorial (MATUSALI *et al.*, 2019).

A transmissão do CHIKV para um humano susceptível ocorre por meio da picada de um mosquito competente da espécie *A. aegypti* ou *A. albopictus* que esteja infectado com o vírus (SILVA *et al.*, 2018; MATUSALI *et al.*, 2019). Esse arbovírus exibe amplo tropismo, replicando-se em diferentes tipos celulares de seu hospedeiro (OLIVER *et al.*, 2019). A primeira molécula descrita como receptor celular do CHIKV foi a proteína de membrana proibitina do tipo 1 (PHB1). Um estudo demonstrou que, ao utilizar anticorpos bloqueadores anti-PHB1, a quantidade de células microgliais infectadas com o CHIKV sofreu uma redução de 20 a 40% quando comparada ao controle. Além disso, em relação ao controle, ocorreu redução de aproximadamente 30% das células infectadas quando ocorreu silenciamento do gene que codifica a proteína PHB1 (WINTACHAI *et al.*, 2012). Por fim, a ligação da glicoproteína viral E2 e da proteína celular PHB1 foi confirmada por imunoprecipitação (SCHNIERLE, 2019). Outras moléculas também foram citadas como cruciais para a entrada do CHIKV nas células hospedeiras humanas, como a molécula de adesão celular MXRA8, a imunoglobulina

de células T, os glicosaminoglicanos, a lectina dependente de cálcio do tipo C (DC-SIGN), a mucina 1 (TIM-1) e TIM-4 (MATUSALI *et al.*, 2019; SCHNIERLE, 2019). Apesar da diversidade de moléculas mencionadas, ainda não há um consenso sobre os receptores celulares definitivos do CHIKV (MATUSALI *et al.*, 2019).

Após a picada de um mosquito infectado, o CHIKV é inserido na pele humana, podendo se replicar em fibroblastos dérmicos, queratinócitos e melanócitos. Ao adentrar a corrente sanguínea, o vírus pode associar-se com glóbulos vermelhos e plaquetas, o que possibilita a transmissão do CHIKV por transfusões de sangue. A carga viral na circulação sanguínea é alta, podendo atingir 10^9 cópias de RNA/mL. As células do sistema linfóide permissivas ao CHIKV são representadas pelos macrófagos derivados de monócitos primários, linfócitos B e células dendríticas plasmocitóides (MATUSALI *et al.*, 2019). A partir da corrente sanguínea, o vírus pode se espalhar para os músculos, articulações, baço, linfonodos, cérebro, fígado, olhos, coração, pulmões e rins (CUNHA; TRINTA, 2017; MATUSALI *et al.*, 2019).

1.2.4. Manifestações Clínicas

Em humanos, o tempo de incubação do CHIKV varia de três a doze dias e indivíduos infectados podem apresentar viremia de até dez dias (SILVA *et al.*, 2018; HIGUERA; RAMÍREZ, 2019). Entre os sintomas principais, destacam-se febre alta (acima de 39 ºC em 92% dos pacientes), erupções cutâneas, artralgia, mialgia, dores de cabeça, náusea, fadiga, vômito e diarréia, podendo desencadear, em casos raros, doenças neurológicas, oculares, renais, hepáticas e respiratórias, miocardite e insuficiência de múltiplos órgãos, o que pode ser fatal (BRAACK *et al.*, 2018; SILVA *et al.*, 2018; MATUSALI *et al.*, 2019; SUKHRALIA *et al.*, 2019). Apesar de geralmente não ser letal, a infecção por CHIKV pode persistir durante anos associada à dor e ao inchaço, principalmente nos pulsos, mãos, tornozelos e pés (BRAACK *et al.*, 2018). Ademais, estudos indicam o aborto como uma das consequências da infecção por CHIKV em gestantes (HIGUERA; RAMÍREZ, 2019).

A doença desencadeada pela infecção por CHIKV pode ser dividida em duas fases: aguda e crônica. A fase aguda ocorre no período correspondente às duas primeiras semanas após o início da doença e se caracteriza pela carga viral alta, linfopenia e trombocitopenia (TANABE *et al.*, 2018). Os sinais clínicos predominantes dessa fase são febre alta, calafrios, poliartralgia, erupções cutâneas, fraqueza e dor de cabeça (MCFEE, 2018; TANABE *et al.*, 2018). Comumente, a ausência desses sintomas é observada após 4 a 7 dias (TANABE *et al.*, 2018). Entretanto, a doença pode evoluir para a fase crônica e consequentemente, a poliartralgia pode persistir por meses ou anos (MCFEE, 2018; TANABE *et al.*, 2018). A persistência da

infecção por CHIKV e a ocorrência de lesões nos tecidos decorrentes da infecção viral são possíveis explicações para um paciente desenvolver a fase crônica da doença (KHONGWICHIT *et al.*, 2021). No período de 36 e 12 meses pós-infecção por CHIKV, casos de poliartralgia foram relatados após surtos ocorrerem na Ilha da Reunião em 2006 e na Itália em 2007, respectivamente (TANABE *et al.*, 2018).

As crianças e os idosos são os grupos que apresentam maior risco para desenvolver a forma mais grave da doença causada pela infecção por CHIKV. No primeiro grupo, casos de hiperpigmentação, eritema, lesões cutâneas bolhosas, convulsões e encefalite são frequentemente observados. Em idosos, a taxa de mortalidade e morbidade aumenta principalmente em pacientes com mais de 75 anos, sendo comum a descrição de insuficiência hepática, colapso cardiovascular, deterioração neurológica e pulmonar e falência de múltiplos órgãos (TANABE *et al.*, 2018; WEAVER *et al.*, 2018).

Os sintomas causados pela infecção por CHIKV são semelhantes aos do ZIKV e DENV, dificultando o diagnóstico com base nos sinais clínicos (MATUSALI *et al.*, 2019; SUKHRALIA *et al.*, 2019). Consequentemente, a subnotificação de casos de infecção por CHIKV é alta devido a diagnósticos incorretos e ausência de notificações, sendo a quantidade de casos provavelmente muito maior do que a registrada (HIGUERA; RAMÍREZ, 2019). Uma diferença marcante entre as infecções por esses arbovírus é o surgimento de sintomas. Enquanto os indivíduos infectados pelo ZIKV e DENV são, em sua maioria, assintomáticos (62% e 75%, respectivamente), a infecção por CHIKV desencadeia sintomas em 72 a 95% dos pacientes. As manifestações clínicas se intensificam quando há coinfecção por CHIKV e ZIKV e/ou DENV (MATUSALI *et al.*, 2019).

A artralgia está presente em aproximadamente 87% dos pacientes infectados por CHIKV, podendo permanecer até 4 meses pós-infecção em 33% dos indivíduos (HIGUERA; RAMÍREZ, 2019; MATUSALI *et al.*, 2019). Esse arbovírus exibe tropismo notável para o tecido ósseo e articular (MCFEE, 2018). As articulações distais são mais afetadas do que as proximais e as células permissivas ao CHIKV são representadas pelos fibroblastos e macrófagos sinoviais. Além da inflamação das articulações, também ocorre danos na cartilagem e formação de áreas de colagenose e fibrose com ocorrência de replicação viral em condrócitos primários. A artrite desencadeada pela infecção por CHIKV também pode ser atribuída à perda óssea e, nesse caso, os osteoblastos são as células permissivas ao vírus (MATUSALI *et al.*, 2019).

Entre as manifestações neurológicas decorrentes da infecção por CHIKV, destacam-se meningite, encefalite, convulsões febris, paralisia flácida aguda e Síndrome de Guillain-Barré.

Estudos indicam a permissividade de células derivadas da crista neural, células cerebrais endoteliais, astrócitos, células microgliais e oligodendrócitos ao arbovírus. Também há evidências que o CHIKV apresenta tropismo preferencial por astrócitos, infectando os neurônios com menor recorrência. Ainda não há um consenso se os sintomas neurológicos são ocasionados pela infecção viral direta do sistema nervoso central ou pelos processos inflamatórios desencadeados pelo arbovírus (MATUSALI *et al.*, 2019).

Além do líquido cefalorraquidiano, verificou-se presença de partículas infecciosas e RNA do CHIKV no tecido ocular de quatro doadores de córnea em uma triagem laboratorial, sugerindo transmissão iatrogênica do vírus por meio do transplante de córnea. No olho humano, o CHIKV infecta os fibroblastos esclerais e da córnea, endotélio da córnea, corpo ciliar, íris e entre as fibras musculares oculares. Os sintomas oculares se caracterizam por dor retro-orbitária, conjuntivite, uveíte, retinite e neurite óptica (MATUSALI *et al.*, 2019).

Além do soro, o CHIKV também é encontrado em outros fluídos corporais de pacientes virêmicos, como saliva, urina e fluído cervico-vaginal. A presença de RNA viral na saliva está relacionada com sangramento oral enquanto, na urina, não há um consenso se o CHIKV está presente devido à replicação em células renais (MATUSALI *et al.*, 2019).

Diversos estudos relataram a presença de RNA de CHIKV no líquido amniótico e na placenta, sugerindo a transmissão materno-fetal desse vírus. A transmissão vertical ocorre em aproximadamente 16% dos casos de gestantes infectadas com o CHIKV (MATUSALI *et al.*, 2019). Em 50% desses casos, ocorre uma doença neonatal sintomática, resultando em altas taxas de morbidade infantil (MARTINEZ *et al.*, 2019; MATUSALI *et al.*, 2019). Os sinais clínicos de recém-nascidos infectados com o CHIKV caracterizam-se por febre, artralgia, irritabilidade, erupções cutâneas, meningoencefalite, insuficiência respiratória aguda e atrasos no desenvolvimento neurológico a longo prazo (WEAVER *et al.*, 2018; MATUSALI *et al.*, 2019). A encefalite neonatal, consequente da transmissão vertical, foi relatada durante uma epidemia que ocorreu no Brasil em 2016 (SILVA *et al.*, 2018). A transmissão do CHIKV de mãe para filho provavelmente ocorre por meio da passagem do vírus da corrente sanguínea materna através de aberturas na placenta. O parto via cesariana não afetar as taxas de transmissão vertical suporta a hipótese de transmissão transplacentária do CHIKV em detrimento da exposição ao arbovírus no canal de parto (MATUSALI *et al.*, 2019).

1.2.5. Diagnóstico

Os procedimentos de diagnóstico do CHIKV concentram-se na identificação do RNA viral, anticorpos IgM e IgG, ou antígenos próprios do vírus (TANABE *et al.*, 2018). O RNA do

CHIKV pode ser detectado no soro do paciente no decorrer dos oito dias iniciais da doença. Os anticorpos específicos para o arbovírus, por sua vez, são observados no final da primeira semana da infecção viral (MCFEE, 2018).

1.2.6. Tratamentos

Como o controle tradicional de vetores encontrou apenas um sucesso limitado na contenção do CHIKV, existe uma necessidade significativa de tratamentos ou vacinas que sejam seguros, eficazes e econômicos para reduzir a disseminação viral e limitar a carga da doença. Atualmente, nenhuma terapia antiviral específica ou vacina licenciada está disponível e somente cuidados paliativos, utilizando analgésicos, antipiréticos, anti-inflamatórios não-esteróides e glicocorticoides sistêmicos, são recomendados para aliviar os sintomas (SILVA *et al.*, 2018; MATUSALI *et al.*, 2019).

A atividade anti-CHIKV de algumas substâncias licenciadas ou não em diversas etapas do ciclo de replicação do vírus foi avaliada em estudos *in vitro* e *in vivo*. Fármacos antivirais licenciados para outros vírus contendo genoma de RNA apresentaram inibição sobre a replicação do CHIKV, como a Ribavirina, Favipiravir, IFN-α, Sofosbuvir e arbidol. A cloroquina, um fármaco antimalárico, inibiu a infecção do CHIKV *in vitro* por meio de interferência na etapa de internalização viral, porém desencadeou aumento da replicação do arbovírus e retardou a resposta imunológica celular e humoral em primatas não-humanos (SILVA *et al.*, 2018; MATUSALI *et al.*, 2019). O fármaco antiparasitário suramina também demonstrou atividade contra o CHIKV por meio da inibição da entrada viral nas células (ALBULESCU *et al.*, 2020). O fármaco anti-hipertensivo telmisartan e o antibiótico novobiocina, por sua vez, bloquearam a atividade de protease da nsP2 do CHIKV, impedindo a infecção viral (TRIPATHI *et al.*, 2020).

A harringtonina e a silimarina são exemplos de substâncias não licenciadas que exibiram ação contra o CHIKV (MATUSALI *et al.*, 2019). O silvestrol, substância encontrada nas plantas do gênero *Aglaia*, demonstrou atividade contra esse arbovírus atuando especificamente na etapa de replicação do genoma do CHIKV (HENSS *et al.*, 2018). O análogo de adenosina 5-iodotubercidina (5-IT) também exibiu ação antiviral sobre a replicação do CHIKV por meio da inibição da proteína não-estrutural nsP1 (MUDGAL *et al.*, 2020). O cloreto de berberina, por sua vez, inibiu a infecção viral ao interferir em etapas tardias do ciclo de replicação do CHIKV (formação do nucleocapsídeo viral) (WAN *et al.*, 2020). Por fim, as imunoglobulinas anti-CHIKV advindas de doadores convalescentes apresentaram atividade antiviral tanto *in vitro* quanto *in vivo* em modelos animais (WEAVER *et al.*, 2018). Embora a taxa de mortalidade

seja modesta, a natureza debilitante e crônica e a carga econômica associada à infecção por CHIKV são considerações importantes para o desenvolvimento de fármacos específicos que atuem no ciclo replicativo do vírus (MATUSALI *et al.*, 2019).

1.3. Peptídeos

Os peptídeos são substâncias orgânicas que se formam a partir da junção de aminoácidos por ligações amida, apresentando peso molecular inferior a 5000–10000 D (MIRSKI *et al.*, 2017). Nas últimas décadas, a descoberta do potencial terapêutico de diversos peptídeos com diferentes alvos biológicos despertou o interesse da comunidade científica nessa classe de substâncias (GOMES *et al.*, 2018; HENNINOT *et al.*, 2018). A ascensão dos peptídeos terapêuticos no mercado farmacêutico é evidenciada pela quantidade crescente de fármacos peptídicos aprovados pela Food and Drug Administration (FDA), representando aproximadamente 10% da produção da indústria farmacêutica (ERAK *et al.*, 2018; GOMES *et al.*, 2018).

Os primeiros estudos com peptídeos envolveram os hormônios naturais, incluindo a insulina que foi o primeiro peptídeo terapêutico, sintetizado em 1921. Com o êxito dos peptídeos naturais, ocorreu investimento crescente no desenvolvimento de peptídeos terapêuticos sintéticos, tais como oxitocina sintética, vasopressina sintética e insulina humana recombinante. Atualmente, existem mais de 80 fármacos peptídicos no mercado (WANG *et al.*, 2022), sendo que, desde 2010, 13 foram aprovados. Em relação aos candidatos a fármacos peptídicos, mais de 170 estão em fase clínica de avaliação e mais de 200 encontram-se em fase pré-clínica (ERAK *et al.*, 2018; LAU; DUNN, 2018; WANG *et al.*, 2022).

Os peptídeos exibem características que favorecem sua abordagem terapêutica. Essa classe de substâncias apresenta biocompatibilidade celular, ou seja, são ligantes naturais para vários receptores de superfície celular, demonstrando alta afinidade e especificidade, o que os torna potenciais candidatos a fármacos com elevada atividade e seletividade (DÍAZ-EUFRACIO *et al.*, 2018; GOMES *et al.*, 2018; HENNINOT *et al.*, 2018). Além disso, os peptídeos apresentam baixa toxicidade já que os seus metabólitos raramente são tóxicos, sendo os aminoácidos os principais produtos de degradação dessa classe de substâncias (CASTEL *et al.*, 2011; ERAK *et al.*, 2018). Consequentemente, os peptídeos demonstram baixa taxa de efeitos colaterais em relação aos medicamentos convencionais (GOMES *et al.*, 2018).

Apesar de exibir uma ampla gama de vantagens em relação aos fármacos convencionais, os peptídeos apresentam limitações farmacocinéticas que restringem o seu uso terapêutico. Essa classe de substâncias demonstra baixa biodisponibilidade resultante da alta biodegradabilidade

por proteases e da baixa absorção epitelial, esta relacionada com a alta polaridade e peso molecular dos peptídeos (ERAK *et al.*, 2018; GOMES *et al.*, 2018; LAU; DUNN, 2018). Além disso, os peptídeos exibem meia-vida curta devido à rápida depuração renal e degradação por peptidases que eliminam e inativam essas substâncias, respectivamente (ERAK *et al.*, 2018; LAU; DUNN, 2018). Por fim, os peptídeos, em casos raros, podem desencadear resposta imunogênica (ERAK *et al.*, 2018).

Uma das estratégias utilizadas para superar as limitações farmacocinéticas dos peptídeos é o desenvolvimento de análogos estruturais que mantenham ou potencializem a bioatividade dos peptídeos e que, principalmente, aperfeiçoem as propriedades farmacêuticas (MIRSKI *et al.*, 2017; GOMES *et al.*, 2018). Diversos estudos demonstram que a conjugação é uma ferramenta relevante no desenvolvimento de novas substâncias (GOMES *et al.*, 2018; HENNINOT *et al.*, 2018; LAU; DUNN, 2018). As subunidades que são conjugadas com peptídeos são classificadas em biomoléculas e moléculas sintéticas que tem como função elevar a meia-vida, biodisponibilidade e/ou solubilidade dos peptídeos (ERAK *et al.*, 2018; LAU; DUNN, 2018). Aproximadamente 30% dos peptídeos que entraram na fase clínica, desde 2010, são conjugados (LAU; DUNN, 2018).

Os peptídeos antimicrobianos (PAMs) representam uma classe de peptídeos que demonstra ação contra diversos microrganismos tanto por atividade direta quanto pela ativação do sistema imunológico do hospedeiro (atividade indireta). Essa classe de peptídeos tem sua origem no sistema imune inato de diferentes organismos e o primeiro relato dessas substâncias ocorreu no início dos anos 80 com a descoberta do peptídeo antimicrobiano magainina em sapos (MIRSKI *et al.*, 2017; GOMES *et al.*, 2018). Os PAMs podem apresentar origem natural, sendo isolados de animais, plantas e microrganismos, ou origem sintética. Os PAMs sintéticos são divididos em duas categorias: (1) análogos baseados nas estruturas de peptídeos naturais, mantendo a subunidade estrutural responsável pela bioatividade e promovendo pequenas alterações na estrutura que visem à melhoria das propriedades farmacocinéticas e/ou da atividade terapêutica; e (2) peptídeos cujo planejamento estrutural não envolveu peptídeos naturais (GOMES *et al.*, 2018).

O peptídeo (p-BthTX-I)$_2$ [(KKYRYHLKPFCKK)$_2$], derivado da toxina Bothropstoxin-I (BthTX-I) presente no veneno da serpente *Bothrops jararacussu*, apresenta atividade antibacteriana contra bactérias gram-positivas e gram-negativas (SANTOS-FILHO *et al.*, 2015). O análogo peptídico (p-BthTX-I)$_2$K, resultado da remoção de Lys^{12}, Lys^{13} e dois resíduos de cisteína e da inserção de um resíduo de lisina na região C-terminal do peptídeo (p-BthTX-I)$_2$ [des-Cys^{11}, Lys^{12}, Lys^{13}-(p-BthTX-I)$_2$K - (KKYRYHLKPF)$_2$K], desencadeou maior

ação antibacteriana que o protótipo $(p\text{-}BthTX\text{-}I)_2$ (SANTOS-FILHO *et al.*, 2022). Outro estudo relatou a atividade antiviral do peptídeo $(p\text{-}BthTX\text{-}I)_2K$ contra o SARS-CoV-2, tendo como alvo a protease viral PL^{pro} (FREIRE *et al.*, 2021).

Dentro dos PAMs, os peptídeos antivirais (PAVs) caracterizam-se como aqueles que exibem atividade contra a infecção de diversos vírus (GOMES *et al.*, 2018; BELTRÁN LISSABET *et al.*, 2019; SCHADUANGRAT *et al.*, 2019). Atualmente, a base de dados de peptídeos antimicrobianos (APD3) compreende 3569 PAMs, dos quais 205 são PAVs (APD3, 2023). A base de dados de peptídeos antivirais validados experimentalmente (PAVdb), por sua vez, contém 2683 AVPs (AVPDB, 2023). Assim como os PAMs, os PAVs apresentam, em sua maioria, sequência curta de aminoácidos (10 a 50 aminoácidos), carga positiva e características tanto hidrofílicas quanto hidrofóbicas (moléculas anfipáticas) (SALA *et al.*, 2018; SCHADUANGRAT *et al.*, 2019).

Os PAVs podem interagir com partículas virais ou atuar em outras diferentes etapas do ciclo de replicação dos vírus para impedir a infecção viral (GOMES *et al.*, 2018; WANG *et al.*, 2022). Os peptídeos inibidores de fusão interagem, por meio de ligações eletrostáticas e hidrofóbicas, com as proteínas de fusão do vírus ou com os lipídeos da membrana celular, bloqueando o processo de fusão da membrana viral e celular e consequentemente, impedindo a entrada do vírus na célula hospedeira (GOMES *et al.*, 2018). O Enfuvirtida ou T20 foi o primeiro tratamento contra o vírus da imunodeficiência humana do tipo 1 (HIV-1) aprovado pela FDA (MATTHEWS *et al.*, 2004). Esse peptídeo sintético inibe a entrada do HIV-1 nas células hospedeiras por meio da interação com a glicoproteína 41 (gp41), presente no envelope viral e uma das responsáveis pela fusão da membrana viral e celular. O peptídeo urumina, isolado de sapos, e P9, derivado da β-defensina de camundongos, bloqueiam o processo de entrada do vírus Influenza A nas células por meio da ligação com a hemaglutinina, glicoproteína presente no envelope viral e responsável pela fusão das membranas viral e endossomal (GOMES *et al.*, 2018; SCHADUANGRAT *et al.*, 2019). O peptídeo C5A, por sua vez, impede a entrada de diversos vírus envelopados em suas células hospedeiras por meio da interação com lipídeos da membrana celular (GOMES *et al.*, 2018). Por fim, o peptídeo Ev37, derivado do veneno de escorpião, inibe a entrada do DENV-2, vírus da hepatite C (HCV), ZIKV e vírus da herpes simples humano do tipo 1 (HSV-1) nas células hospedeiras ao desencadear alcalinização de organelas ácidas, bloqueando a fusão das membranas viral e endossomal (LI *et al.*, 2019).

Além dos peptídeos inibidores de fusão, também existem os peptídeos que bloqueiam a replicação do vírus (GOMES *et al.*, 2018; SCHADUANGRAT *et al.*, 2019). A protegrina-1, um peptídeo cíclico derivado de glóbulos brancos de suínos, exerce atividade contra o DENV

por meio da inibição da protease viral NS2B-NS3Apro, essencial no processo de replicação do vírus (SCHADUANGRAT *et al.*, 2019). Os fármacos peptídicos boceprevir e telaprevir, aprovados em 2011 como terapia contra o HCV, inibem a atividade da serina protease NS3/4A viral, impedindo a etapa de replicação do HCV nas células hospedeiras (WANG *et al.*, 2022).

Além de interferência na internalização e replicação viral, os PAVs apresentam outros mecanismos de ação antivirais, como disrupção do envelope de vírus envelopados, bloqueio da adsorção e liberação viral nas células hospedeiras, inibição total ou parcial da montagem da partícula viral, entre outros (GOMES *et al.*, 2018; SCHADUANGRAT *et al.*, 2019). Além dos vírus mencionados, a atividade dos PAVs também foi relatada contra outros vírus, como CHIKV (SHOJI-KAWATA *et al.*, 2013; ROTHAN *et al.*, 2014), vírus da raiva (REAL *et al.*, 2004), SARS-CoV e coronavírus relacionado à síndrome respiratória do Oriente Médio (MERS-CoV) (ZHAO *et al.*, 2016).

O Hecate (FALALKALKKALKKLKKALKKAL) é um peptídeo sintético que apresenta carga líquida positiva e características anfipáticas, formando uma estrutura em α-hélice (BATISTA *et al.*, 2018). Esse peptídeo exibe diversas atividades biológicas, destacando-se a antiviral e antitumoral (SANCHES *et al.*, 2015; BATISTA *et al.*, 2018). O mecanismo de ação do Hecate caracteriza-se pela interação do peptídeo com biomembranas por meio da ligação com fosfolipídeos e consequente intercalação na bicamada lipídica. Essa interação provoca uma instabilidade química na membrana, alterando sua fluidez e desencadeando sua disrupção (SATO; FEIX, 2006). Como o envelope viral se origina da bicamada lipídica das membranas celulares, o peptídeo Hecate exerce sua atividade antiviral por meio da interação com o envelope de vírus envelopados. Essa ligação desencadeia a ruptura da estrutura do envelope ou desestabilização na formação deste na partícula viral, resultando em inibição do processo de fusão das membranas viral e celular na etapa de entrada viral ou bloqueio da liberação do vírus a partir da célula hospedeira, respectivamente (BATISTA *et al.*, 2018).

O ácido gálico ou ácido 3,4,5-trihidroxibenzóico (AG) ($C_7H_6O_5$) é uma substância fenólica encontrada em abundância nos vegetais, sendo isolado de diversas espécies de plantas, como *Quercus* spp. e *Punica* spp.. Essa substância apresenta vasta gama de aplicações na indústria farmacêutica e exibe ampla atividade biológica, destacando a antiviral, antibacteriana e antitumoral (KAHKESHANI *et al.*, 2019; ZHANG *et al.*, 2019). Em relação à ação antiviral, o AG mostrou-se ativo contra o HSV-1 em células renais de macaco verde africano (Vero) (EL-AGUEL *et al.*, 2022) e também reduziu a infecção do alfavírus o'nyong-nyong (ONNV) em células de fibroblastos de pele humana (HSF) (SEPTEMBRE-MALATERRE *et al.*, 2021). O AG exibe atividade antiviral por meio de diferentes mecanismos de ação, tais como efeito

virucida, ação profilática e interferência na adsorção, internalização e replicação do vírus nas células hospedeiras (BATOOL *et al.*, 2018; YOU *et al.*, 2018; KAHKESHANI *et al.*, 2019).

Nosso grupo avaliou a atividade anti-HCV de cinco análogos do peptídeo Hecate. Esse peptídeo conjugado ao AG (AG-Hecate) representou o análogo mais ativo, inibindo as principais etapas do ciclo de replicação do HCV. Na concentração de 20 µM, o AG-Hecate inibiu aproximadamente 85% e 95% da replicação e entrada do HCV, respectivamente, em células Huh-7.5 derivadas de carcinoma hepatocelular humano. Em concentrações igual ou menores que 20 µM, esse análogo provocou inibição de 50% da liberação do HCV de células Huh-7.5. O estudo também constatou que as partículas virais liberadas das células tratadas com AG-Hecate não foram capazes de infectar novas células Huh 7.5, indicando que o análogo desencadeou danos na partícula do HCV que resultaram em bloqueio da infectividade do vírus. Além do efeito antiviral, o AG-Hecate demonstrou baixa toxicidade em células Huh-7.5, sendo o análogo mais seletivo dentre os cinco testados. As propriedades antivirais do AG-Hecate encorajam a sua utilização como protótipo para o desenvolvimento de novos antivirais de amplo espectro (BATISTA *et al.*, 2018).

2. OBJETIVOS

2.1. Objetivo Geral

Avaliar peptídeos sintéticos quanto à atividade anti-CHIKV e em relação ao efeito de inibição sobre cada etapa do ciclo de replicação do vírus.

2.2. Objetivos Específicos

(A) Avaliar a toxicidade dos peptídeos em células de fibroblastos renais de hamster recém-nascido (BHK-21) e células epiteliais de hepatocarcinoma humano (Huh-7);
(B) Avaliar a atividade antiviral dos peptídeos sobre o ciclo de replicação completo do CHIKV;
(C) Avaliar os peptídeos quanto à ação protetiva para as células hospedeiras contra a infecção viral;
(D) Avaliar os peptídeos em relação ao efeito virucida;
(E) Avaliar os peptídeos quanto à atividade inibitória sobre a entrada viral (adsorção e internalização);
(F) Avaliar os peptídeos em relação à ação inibitória sobre as etapas de pós-entrada viral.

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Capítulo II

Artigo Científico I

The synthetic peptide GA-Hecate and its analogs inhibit multiple steps of the CHIKV infection cycle *in vitro*

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ABSTRACT

Chikungunya virus (CHIKV) belongs to the *Alphavirus* genus and is responsible for significant outbreaks worldwide. Currently, there is no approved antiviral therapy against CHIKV. Bioactive peptides have great potential for new drug development. Here, we evaluated the antiviral activity of the synthetic peptide GA-Hecate and its analogs PSSct1905 and PSSct1910 against CHIKV infection. Initial screening showed that all three peptides inhibited the CHIKV replication cycle in BHK-21 and Huh-7 cells. GA-Hecate and its analog PSSct1905 were the most active, demonstrating suppression of viral infection by more than 91%. The analog PSSct1905 exhibited a protective effect in cells against CHIKV infection. We also observed that analogs PSSct1905 and PSSct1910 affected CHIKV entry into both cell lines, inhibiting viral attachment and internalization. Finally, all tested compounds presented antiviral activity on the postentry steps of CHIKV infection in all cells evaluated. In conclusion, this study highlights the potential of the peptide GA-Hecate and its analogs as novel anti-CHIKV compounds targeting different stages of the viral replication cycle, warranting the development of GA-Hecate-based compounds with broad antiviral activity.

Keywords: Chikungunya. Peptides. Analogs. Antiviral.

INTRODUCTION

Chikungunya virus (CHIKV) is an arbovirus that belongs to the genus *Alphavirus* (family *Togaviridae*). The virions have a single-stranded and positive-sense RNA genome of approximately 11.5 kb in size, which is polyadenylated at the 3' end and has a 7-methylguanosine cap at the 5' end. The genome contains two open reading frames (ORFs): (1) a 5' ORF that encodes a nonstructural polyprotein that is processed into four nonstructural proteins (nsP1, 2, 3, and 4), which are parts of a viral replication complex, and (2) a 3' ORF encoding a polyprotein that is processed into five main structural proteins (C, E1, E2, E3, and 6K) [1,2]. The genetic material of CHIKV is surrounded by a capsid (240 copies of C) with icosahedral symmetry, forming the viral nucleocapsid. The CHIKV particle has an envelope derived from the plasma membrane of host cells to which 80 spikes formed by trimers of glycoproteins E1 and E2 are anchored [3,4].

CHIKV was first reported during an outbreak that occurred in Tanzania in 1953. Currently, this arbovirus is fully adapted to the urban transmission cycle, raising concern in many tropical and more temperate regions [5]. Although CHIKV continues to cause significant outbreaks around the world to date, there are no specific antiviral treatments or licensed vaccines against this viral infection [6]. Several antiviral strategies against CHIKV are being evaluated, mainly *in vitro*, that can affect critical steps of the viral replication cycle, such as binding and entry, viral genome replication, functionality of viral proteins, virion formation and infectivity [7-11].

Antiviral peptides that interact with virus particles or target other critical steps of viral infection could potentially be used as treatment or prophylaxis of virus infection [12,13]. Most antiviral peptides are short (10 to 50 amino acid residues), have a positive charge, and have both hydrophilic and hydrophobic characteristics [14,15]. These peptides usually act at different steps of the virus replication cycle [16]. For example, the peptide (p-BthTX-I)$_2$K derived from *Bothrops jararacussu* snake impaired CHIKV entry into baby hamster kidney fibroblast cells by inhibiting the attachment and internalization steps. Additionaly, the same peptide also inhibited Zika virus (ZIKV) infection by interfering with the postentry stages of the viral replication cycle, reducing the viral protein NS3, which is essential for ZIKV replication [17]. Antiviral peptides have other mechanisms of action, such as disruption of the virus envelope, blocking release in host cells, and total or partial inhibition of viral particle assembly [8,15,16].

Different studies have demonstrated that peptide conjugation is a relevant strategy for the development of new substances and to increase their biological activity [12,18,19]. The peptide Hecate (FALALKALKKALKKLKKALKKAL-CONH$_2$) is a synthetic peptide that has a net

positive charge and amphipathic characteristics, exhibiting many biological activities, with emphasis on antiviral and antitumor activity [20,21]. Gallic acid (GA) ($C_7H_6O_5$) is a phenolic compound found in abundance in plants that exhibits antiviral, antibacterial, and antitumor activities [22,23]. Our group has evaluated the anti-hepatitis C virus (HCV) activity of five analogs of Hecate in human hepatocellular carcinoma-derived Huh-7.5 cells. The peptide conjugated to GA (GA-Hecate) represented the most active compound, inhibiting the main stages of the HCV replication cycle. Thus, the antiviral properties of GA-Hecate encourage its use as a prototype for the development of new antivirals [20].

This study aimed to evaluate the potential of GA-Hecate and its analogs PSSct1905 (GA-FALALKALKKALKKL-COOH) and PSSct1910 (4-(dimethylamino)-benzoic acid-FALALKALKKALKKLKKALKKAL-$CONH_2$) as antivirals against CHIKV; the study was performed by testing the effects of these peptides on different steps of the CHIKV infectious cycle.

MATERIALS AND METHODS

Peptides

GA-Hecate and its analogs PSSct1905 and PSSct1910 were prepared on a TRIBUTE-UV automatic synthesizer via solid phase peptide synthesis (SPPS) using the standard Fmoc protocol (9-fluorenylmethyloxycarbonyl) on a Rink-MBHA resin for the peptides GA-Hecate and PSSct1910 and Fmoc-Leu-Wang resin for PSSct1905 [21]. The identities of the peptides were confirmed by electrospray ionization mass spectrometry, and their purities were higher than 95%. To perform the biological experiments, the peptides were dissolved in sterile water and stored at –150 ºC.

Cells

Baby hamster kidney fibroblast cells (BHK-21; ATCC CCL-10) and human hepatocarcinoma epithelial cells (Huh-7) were cultured in Dulbecco's modified Eagle's medium (DMEM, Cultilab, São Paulo, BR) supplemented with 10% fetal bovine serum (FBS, Cultilab, São Paulo, BR) and 1% penicillin (10,000 IU/mL)/streptomycin (P/S) (10 mg/mL) (Gibco – Thermo Fisher Scientific, Massachusetts, USA). The cells were maintained in a humidified incubator at 37 °C in 5% CO_2.

Virus

The CHIKV expressing *nanoluciferase* reporter (CHIKV-NLuc), used for the antiviral assays, is based on the CHIKV isolate LR2006OPY1 (East/Central/South African genotype). In the infectious plasmid, the cDNA of CHIKV-NLuc was placed under the control of the CMV promoter [24]. To rescue CHIKV-NLuc, 1×10^5 BHK-21 cells seeded in 24-well culture plates were transfected with 1 µg of CHIKV-NLuc plasmid DNA using Lipofectamine 2000 (Thermo Fisher Scientific, Massachusetts, USA) and OPTI-MEM (reduced serum medium) (Gibco – Thermo Fisher Scientific, Massachusetts, USA), as previously described [25]. The supernatant was collected at 72 hours posttransfection (h.p.t.) and stored at –80 °C.

The viral titers were determined by plaque assay, following the previously described protocol [25]. BHK-21 cells (1×10^5) were seeded in 24-well plates. Twenty-four hours later, the cells were infected with tenfold serial dilutions of CHIKV-NLuc and incubated for 1 hour at 37 °C. After this, the inoculums were removed and replaced with fresh medium supplemented with 1% P/S (Gibco – Thermo Fisher Scientific, Massachusetts, USA), 1% FBS (Cultilab, São Paulo, BR) and 2% carboxymethylcellulose (CMC) (Sigma–Aldrich, Missouri, USA). Infected cells were incubated for 48 hours in a humidified 5% CO_2 incubator at 37 °C, followed by fixation with 10% formaldehyde (Merck, Darmestádio, DE) and staining with 1% violet crystal (Merck, Darmestádio, DE). The viral foci were counted to determine the viral titer, which was presented in plaque-forming units per milliliter (PFU/mL).

Evaluation of the cytotoxicity profile of the peptides

Cytotoxicity in BHK-21 and Huh-7 cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [20]. The cells (5×10^3 per well) were seeded in 96-well culture plates (TPP, Trasadingen, SWI). After 24 hours, the cells were incubated with the peptides at concentrations of 1.6, 3.1, 6.3, 12.5, 25, 50, and 100 µM for 24 hours. Then, the medium containing the peptides was removed, and 100 µL of MTT (Sigma–Aldrich, Missouri, USA) diluted in DMEM (Cultilab, São Paulo, BR) (1 mg/mL) was added to each well of the plate (1 mg/mL). After 30 minutes of incubation at 37 °C, the medium containing MTT (Sigma–Aldrich, Missouri, USA) was removed, and 100 µL of dimethylsulfoxide (DMSO) (Synth, São Paulo, BR) was added to the cells. The plate was agitated at 200 rpm. After 5 minutes, the absorbance was measured at a wavelength of 572 nm on a plate reader (FLUOstar Omega/BMG LABTECH, Ortenberg, DE). The 50% cytotoxic

concentration (CC_{50}) values were calculated using GraphPad Prism 5.0 software (GraphPad Software, California, USA).

Analysis of the activity of the CHIKV-NLuc-encoded reporter

Measurement of the activity of the virus-encoded NLuc reporter was used to evaluate the antiviral activity of the peptides against CHIKV-NLuc. Cells were infected with CHIKV-NLuc, and the supernatant was aspirated 16 hours post-infection (h.p.i.), and each well of the plate was washed with PBS (phosphate-buffered saline) solution. Then, 30 µL of Renilla Luciferase Assay Lysis Buffer (Promega, Wisconsin, USA) was added to the cells. After 30 minutes, 50 µL of substrate for Renilla Luciferase (Renilla Luciferase Assay Reagent, Promega, Wisconsin, USA) was automatically injected in the wells into the plate reader (FLUOstar Omega/BMG LABTECH, Ortenberg, DE), and the light intensity reading was performed. The values obtained are shown as the percentage of values of protein NanoLuciferase (NLuc) activity in the control (samples treated with sterile water).

Evaluation of the activity of peptides against the CHIKV replication cycle

The effects of the peptides on the CHIKV infectious cycle were analyzed as previously described [26]. BHK-21 and Huh-7 cells (1×10^4 per well) were seeded in 96-well white culture plates (Greiner Bio-one, São Paulo, BR) for 24 hours. Then, the virus at a multiplicity of infection (MOI) of 0.1 and the peptides at the maximum nontoxic concentration (MNTC) were simultaneously added to the cells. The plate was incubated at 37 °C for 16 hours, and then antiviral activity against CHIKV was evaluated through NLuc activity. The peptides that exhibited antiviral activity at the MNTC were tested at concentrations of 1.6, 3.1, 6.3, 12.5, 25, 50, and 100 µM to determine the 50% effective concentration (EC_{50}) for CHIKV inhibition, which was calculated using GraphPad Prism 5.0 software (GraphPad Software, California, USA). The selectivity index (SI) was calculated by the ratio CC_{50}/EC_{50}.

Analysis of the protective effect of the peptides against CHIKV infection in the pretreatment assay

The protective effect of the peptides against CHIKV infection was evaluated as previously described [26]. BHK-21 and Huh-7 cells (1×10^4 per well) were seeded in 96-well white culture plates (Greiner Bio-one, São Paulo, BR). After 24 hours, the cells were treated with peptides at the MNTC for 1 hour at 37 °C. Then, the supernatant was removed, each well of the plate was washed twice with PBS, and the virus at an MOI of 0.1 was added to the cells. The plate was

incubated at 37 °C, and the protective effect against CHIKV infection was evaluated 16 h.p.i. by measurement of NLuc activity.

Evaluation of the effect of the peptides on the extracellular CHIKV particles

The virucidal activity of the peptides against CHIKV virions was investigated as previously described [27]. BHK-21 and Huh-7 cells (1×10^4 per well) were seeded in 96-well white culture plates (Greiner Bio-one, São Paulo, BR). A total of 5×10^4 PFU of virus was incubated with the MNTC of peptides at 37 °C for 1 hour, after which the viral inoculum was added to the cells. After 1 hour, the supernatant was aspirated, the wells were washed twice with PBS, and DMEM (Cultilab, São Paulo, BR) supplemented with 2% FBS (Cultilab, São Paulo, BR) was added to the cells. The plate was incubated at 37 °C for 16 h.p.i. The viral infection rate was quantified by measuring NLuc activity.

Analysis of the peptides in the CHIKV entry steps

The peptides' action on CHIKV entry into the cells was analyzed as previously described [25,26]. BHK-21 and Huh-7 cells (1×10^4 per well) were seeded in 96-well white culture plates (Greiner Bio-one, São Paulo, BR) for 24 hours. Then, the cells were infected with the virus at an MOI of 0.1 and treated with the peptides at the MNTC simultaneously for 1 hour at 37 °C. Subsequently, the supernatant was removed, the cells were washed twice with PBS, and DMEM (Cultilab, São Paulo, BR) with 2% FBS (Cultilab, São Paulo, BR) was added to each well. The plate was incubated at 37 °C, and the antiviral effect against CHIKV was evaluated 16 h.p.i. by measurement of NLuc activity.

Analysis of the peptides on the CHIKV attachment to the cells

The effect of the peptides on CHIKV attachment to cells was evaluated as previously described [25,26,28]. BHK-21 and Huh-7 cells (1×10^4 per well) were seeded in 96-well white culture plates (Greiner Bio-one, São Paulo, BR). After 24 hours, the plate was incubated at 4 °C for 15 minutes, and the cells were infected with the virus at an MOI of 0.1 and treated with the peptides at the MNTC simultaneously. The plate was incubated at 4 °C for 1 hour. At 4 °C, virions interact with receptors on the cell membrane, but the viral particle is not able to enter the cell [25]. Then, the supernatant was aspirated, the wells were washed twice with PBS, and DMEM (Cultilab, São Paulo, BR) supplemented with 2% FBS (Cultilab, São Paulo, BR) was added. The plate was incubated at 37 °C, and the antiviral activity against CHIKV was evaluated 16 h.p.i. by measurement of NLuc activity.

Analysis of the peptides on CHIKV internalization

The activity of the peptides on CHIKV internalization in cells was analyzed as previously described [28]. BHK-21 and Huh-7 cells (1×10^4 per well) were seeded in 96-well white culture plates (Greiner Bio-one, São Paulo, BR). After 24 hours, the plate was incubated at 4 °C for 15 minutes, and the cells were infected with the virus at an MOI of 0.1 for 1 hour at 4 °C. Then, the supernatant was removed, and the cells were washed twice with PBS and treated with the peptides at the MNTC for 1 hour at 37 °C. Subsequently, the supernatant was aspirated, the cells were again washed twice with PBS, and DMEM (Cultilab, São Paulo, BR) with 2% FBS (Cultilab, São Paulo, BR) was added. The plate was incubated at 37 °C, and the inhibitory action on CHIKV was evaluated 16 h.p.i. by measurement of NLuc activity.

Evaluation of the peptides on the CHIKV postentry steps in cells

The peptides' action on the postentry stages of CHIKV in cells was evaluated as previously described [25,26]. BHK-21 and Huh-7 cells (1×10^4 per well) were seeded in 96-well white culture plates (Greiner Bio-one, São Paulo, BR). After 24 hours, the cells were infected with the virus at an MOI of 0.1. After 1 hour at 37 °C, the supernatant was removed, and the cells were washed twice with PBS and treated with the peptides at the MNTC for 16 h.p.i. The plate was incubated at 37 °C, and CHIKV replication was quantified by measuring NLuc activity.

Statistical analysis

All the described experiments were performed in three independent events, each of which was carried out in three (cytotoxicity) and four (antiviral activity) technical replicates. The CC_{50} and EC_{50} values were determined by nonlinear regression of the dose–response curves (Log [peptide] × response). The statistical significance was determined employing the paired Student's t test for parametric results and the Mann–Whitney test for nonparametric data using GraphPad Prism 5.0 software (GraphPad Software, California, USA). A p value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Cytotoxicity of the peptides

The structures of the peptide GA-Hecate and its analogs PSSct1905 and PSSct1910 are presented in Figure 1. The physicochemical properties and biological activities of these peptides are summarized in Table 1. The cytotoxicity of the peptides GA-Hecate, PSSct1905, and PSSct1910 was evaluated in BHK-21 and Huh-7 cells. We defined the MNTC, expressed in

µM, as the highest peptide concentration that kept at least 80% of BHK-21 and Huh-7 cells viable within 24 hours.

All peptides had higher CC_{50} values for Huh-7 cells than for BHK-21 cells. The MNTC of GA-Hecate and its analog PSSct1905 was 12.5 µM or higher in these cell types (Figures S1A, B, D, and E). Compared to GA-Hecate, the peptide PSSct1905 lacks the last eight amino acids in the C-terminus, a modification made on the basis of a previous observation that these amino acids were susceptible to degradation by blood proteases, decreasing the stability of the compound (data not shown). Based on our analysis, this modification resulted in a modest (approximately 2-fold) increase in the CC_{50} value of the compound; the difference was more prominent in BHK-21 cells. The analog PSSct1910 was considerably more cytotoxic, with CC_{50} values of 5.4 µM and 7.7 µM for BHK-21 and Huh-7 cells, respectively. For both cell lines, the MNTC was 1.6 µM (Figures S1C and F). These data indicate that the only structural change that promoted higher cytotoxicity was the replacement of GA by the (4-(dimethylamino)-benzoic acid) subunit at the N-terminal end of the peptide GA-Hecate. Additional studies of the structure-activity-relationship should be performed to better understand the role of these modifications in the cytotoxic effects of these compounds.

Figure 1. Structures of the peptide GA-Hecate and its analogs PSSct1905 and PSSct1910

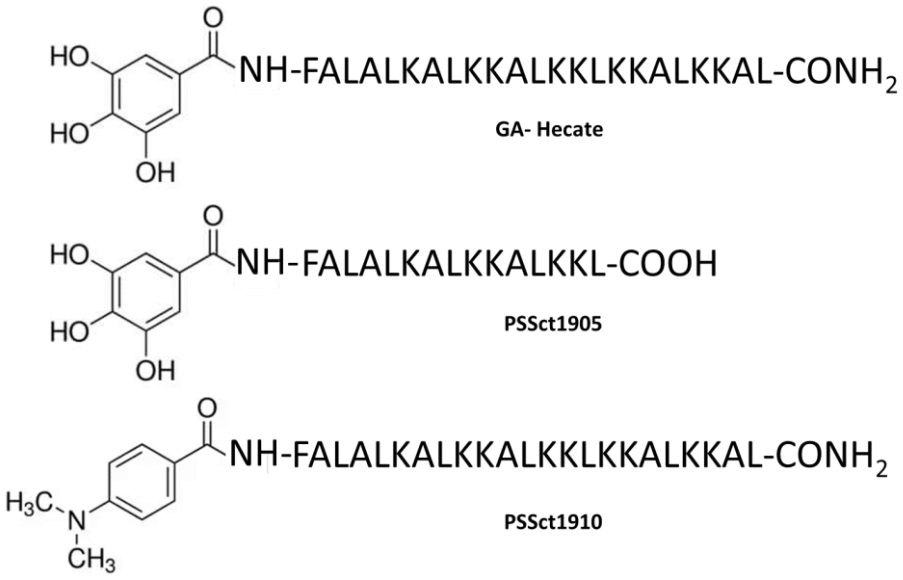


Table 1. General characteristics and biological properties for the cationic peptides GA-Hecate, PSSct1905, and PSSct1910. **MW:** molecular weight. **MNTC:** maximum nontoxic concentration. **CC_{50}:** 50% cytotoxic concentration. **EC_{50}:** 50% effective concentration. **SI:** selectivity index.

					BHK-21 cells			**Huh-7 cells**		
Peptide	**Length**	**MW**	**Net Charge**	**MNTC (µM)**	**CC_{50} (µM)**	**EC_{50} (µM)**	**SI**	**CC_{50} (µM)**	**EC_{50} (µM)**	**SI**
GA-Hecate	23	2688.43	+9	12.5	23.3	5.7	4.1	221	4.5	49.1
PSSct1905	15	1808.25	+4	12.5	56.5	8.4	6.7	205	7.1	28.9
PSSct1910	23	2684.17	+9	1.6	5.4	1.1	4.9	7.7	0.2	38.5

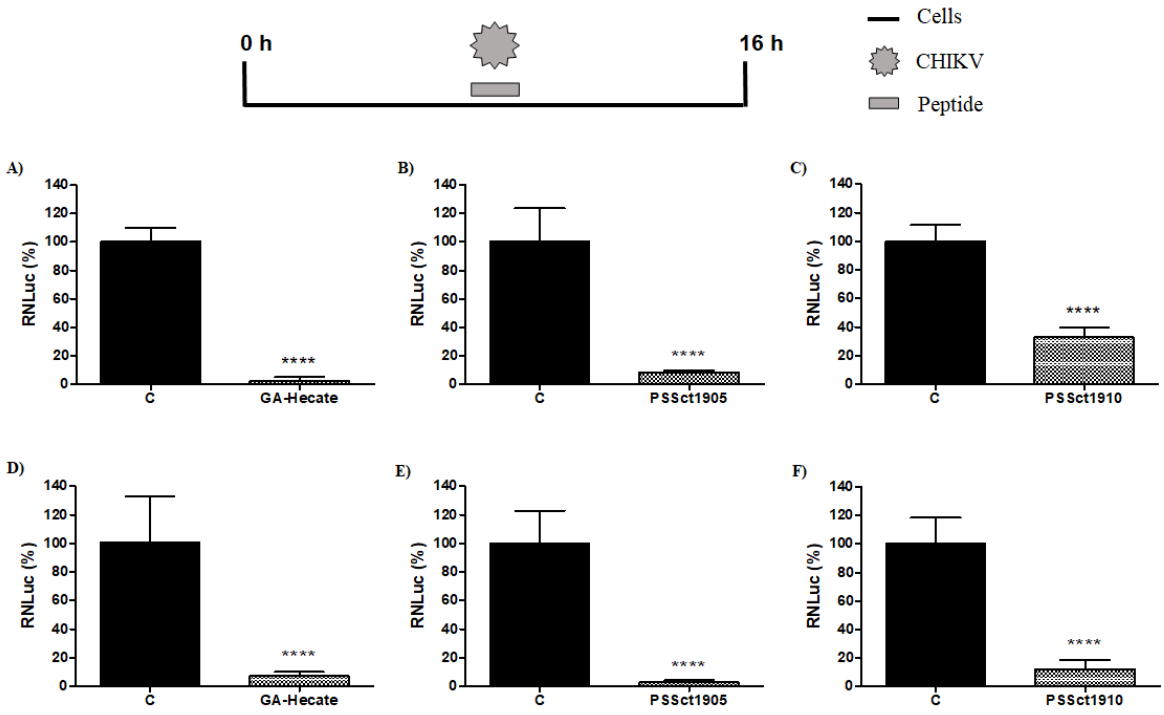
The peptides strongly impaired the replication cycle of CHIKV

The peptides GA-Hecate, PSSct1905, and PSSct1910 were tested at the MNTC in BHK-21 and Huh-7 cells infected with CHIKV-NLuc (MOI 0.1) for 16 hours. All three peptides exhibited significant antiviral effects against the replication cycle of CHIKV. GA-Hecate was the most active, causing an inhibition of viral replication of 97.7% ($p \leq 0.0001$) in BHK-21 cells and 93.6% ($p \leq 0.0001$) in Huh-7 cells when compared to the control (Figures 2A and D). Among the analogs, PSSct1905 also showed a high level of inhibition of the CHIKV replicative cycle; the effect was slightly more prominent in Huh-7 cells (97.3% inhibition; $p \leq 0.0001$) than in BHK-21 cells (91.7% inhibition; $p \leq 0.0001$) (Figures 2B and E). The similar activities imply that removal of KKALKKAL amino acids from the C-terminal end of GA-Hecate has minimal effect on the antiviral activity of the compound. On the other hand, the replacement of the GA by the group with (4-(dimethylamino)-benzoic acid at the N-terminal end of the peptide GA-Hecate negatively impacted its inhibitory potential; peptide PSSct1910, harboring such a modification, resulted in 67.2% inhibition ($p \leq 0.0001$) of CHIKV replication in BHK-21 cells and 87.8% inhibition ($p \leq 0.0001$) in Huh-7 cells (Figures 2C and F). However, it should be noted that due to its increased cytotoxicity, this compound was also used at much lower concentration than other two inhibitors. Indeed, PSSct1910 treatment resulted in the lowest EC_{50} values, with a higher inhibitory effect (approximately 5-fold) in Huh-7 cells (EC_{50} = 0.2

μM) than in BHK-21 cells (EC_{50} = 1.1 μM). Thus, it may still be an efficient inhibitor. All peptides had a higher SI in Huh-7 cells than in BHK-21 cells. GA-Hecate was the most selective in Huh-7 cells, resulting in an SI value of 49.1.

The peptide Hecate can bind with lipids, disrupting lipid bilayers [29]. Hecate's ability to bind with lipids can trigger changes in the fluidity of the virus envelope, cause disturbance in viral envelope formation, and interfere with viral fusion and/or egress in host cells [30]. Indeed, Hecate showed antiviral activity against an unrelated enveloped virus, herpes simplex virus (HSV-1), infection in African green monkey kidney cells (Vero) by inhibiting cell fusion [31]. GA also inhibited the replication cycle of HSV in Vero cells [32]. It has also been demonstrated that this compound significantly reduced the infection of o'nyong-nyong virus (ONNV), an alphavirus closely related to CHIKV, in human skin fibroblast (HSF) cells [33]. GA exhibits antiviral activity through different mechanisms of action, such as virucidal and protective effects and interference with virus entry and replication in host cells [22,34,35]. GA-Hecate inhibited the efficiency of multiple steps in the HCV replication cycle in Huh-7.5 cells [20]. These data indicate that GA-Hecate and its analogs may impair the CHIKV replication cycle due to interference with more than one step of the CHIKV infection cycle. For this reason, we decided to evaluate which phase of the CHIKV replication cycle these peptides could inhibit.

Figure 2. Inhibitory effect of the peptides on the CHIKV replication cycle in BHK-21 and Huh-7 cells. The percentages were expressed in relation to NLuc activity in cells treated with the control (**C**, sterile water). Luminescence signals were measured in three independent experiments, each performed in quadruplicate, and data are presented as the mean ± standard deviation (SD). **(A)** GA-Hecate at the MNTC (12.5 µM) in BHK-21 cells. **(B)** PSSct1905 at the MNTC (12.5 µM) in BHK-21 cells. **(C)** PSSct1910 at the MNTC (1.6 µM) in BHK-21 cells. **(D)** GA-Hecate at the MNTC (12.5 µM) in Huh-7 cells. **(E)** PSSct1905 at the MNTC (12.5 µM) in Huh-7 cells. **(F)** PSSct1910 at the MNTC (1.6 µM) in Huh-7 cells. ******:** $p \leq 0.0001$.



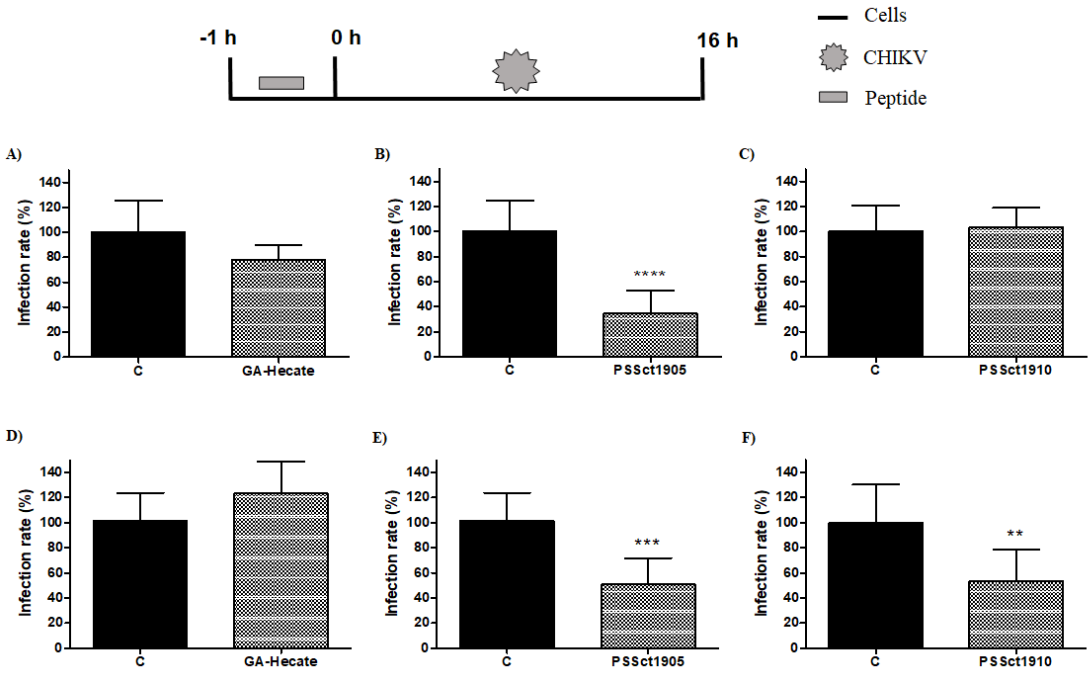
Antiviral effect of pretreatment with the peptides against CHIKV

To evaluate the protective effect of pretreatment of cells against CHIKV infection, BHK-21 and Huh-7 cells were treated with the peptides at the MNTC for 1 hour, washed with PBS and infected with CHIKV-NLuc (MOI 0.1) for 16 hours. Pretreatment of cells with GA-Hecate did not result in detectable protection against CHIKV infection (Figures 3A and D), while its analog PSSct1905 was able to significantly protect BHK-21 and Huh-7 cells, reducing CHIKV infection by 65.4% ($p \leq 0.0001$) and 50.6% ($p \leq 0.001$), respectively (Figures 3B and E). Pretreatment with PSSct1910 had a protective effect against CHIKV in Huh-7 cells (inhibition by 46.1%; $p \leq 0.01$) but not in BHK-21 cells (Figure 3C and 3F).

Taken together, pretreatment of cells with peptides revealed that protection of cells by prophylactic treatment is not the main mechanism of action of these antiviral peptides.

Nevertheless, some modest antiviral activity was observed. CHIKV infection starts with the binding of virus particles to the cellular surface, triggering attachment and internalization [36,37]. The viral glycoprotein E2 is responsible for interacting with cell receptors, including Mxra8, the main receptor for several arthritogenic alphaviruses, including CHIKV [38,39]. The CHIKV cell receptor is ubiquitously expressed in several species and cell types [40,41]. Importantly, the binding of CHIKV virions is facilitated by several other cellular components. Glycosaminoglycans (GAGs), prohibitin (PHB) and phosphatidylserine (PtdSer)-mediated virus entry-enhancing receptors (PVEERs) are cited in the literature as factors of CHIKV attachment in mammalian cells [42-44]. Interestingly, the accumulation of positively charged residues in the E2 protein of alphaviruses has been demonstrated to be common adaptation for growth in cell culture as these residues confer binding to negatively charged molecules (such as GAGs) on the surface of mammalian cells [45-48]. All tested peptides also have positive charges and, probably, in the pretreatment, the positive charges facilitate their binding to the cell. This binding may inhibit the interaction between the E2 glycoprotein and molecules on the cell surface and consequently also the CHIKV attachment and internalization in the host cell. However, alone the positive charge of peptides could not explain all observed effects as GA-Hecate is also positively charged yet offers no protection. It is plausible that the size difference between this peptide and the analog PSSct1905 may be responsible for different interactions with CHIKV attachment factors/receptors and these differences alter ability of compound to protect cells against infection.

Figure 3. Protective effect of the peptides against CHIKV infection. Data were analyzed as described for Figure 2. Luminescence signals were measured from three independent experiments, each performed in quadruplicate, and data are presented as the mean ± SD. **(A)** GA-Hecate in BHK-21 cells. **(B)** PSSct1905 in BHK-21 cells. **(C)** PSSct1910 in BHK-21 cells. **(D)** GA-Hecate in Huh-7 cells. **(E)** PSSct1905 in Huh-7 cells. **(F)** PSSct1910 in Huh-7 cells. ****:** $p \leq 0.01$; *****:** $p \leq 0.001$; ******:** $p \leq 0.0001$.



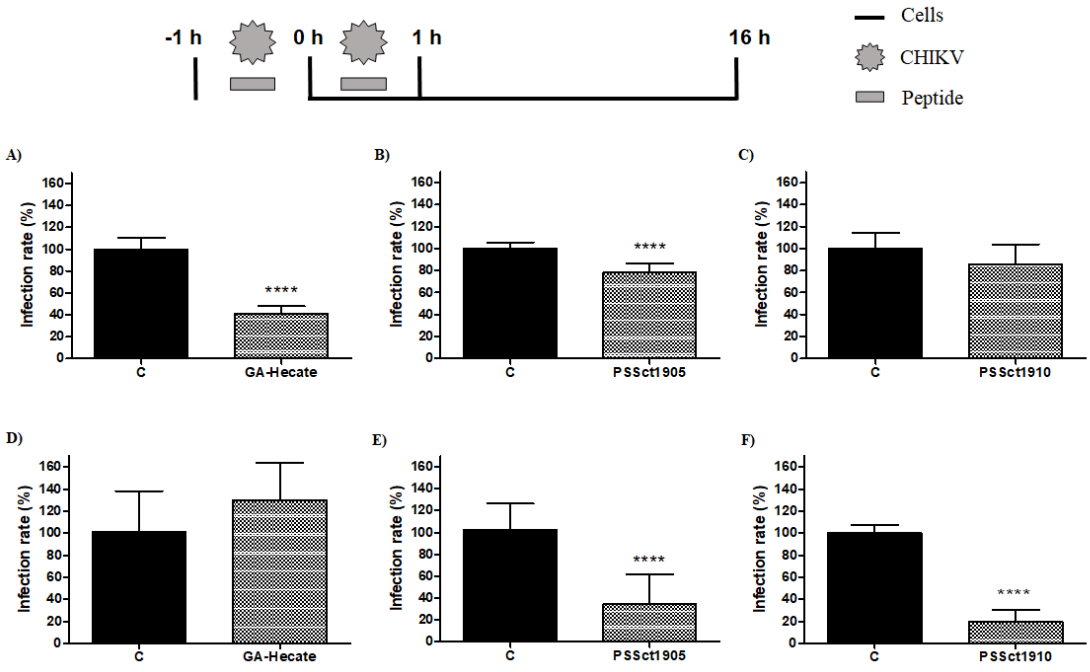
Virucidal effect of the peptides on CHIKV virions

To investigate the ability of peptides GA-Hecate, PSSct1905, and PSSct1910 to inactivate extracellular CHIKV virions, the virus particles were treated with the peptides at the MNTC for 1 hour and used for infection of BHK-21 and Huh-7 cells. In this assay, the peptides GA-Hecate and PSSct1905 reduced infection in BHK-21 cells, showing inhibitory effects of 59.4% ($p \leq 0.0001$) and 21.4% ($p \leq 0.0001$), respectively (Figures 4A and B). In Huh-7 cells, GA-Hecate did not inhibit viral infection (Figure 4D), while treatment with PSSct1905 resulted in 68% ($p \leq 0.0001$) inhibition (Figure 4E). The peptide PSSct1910, on the other hand, only presented virucidal activity when treated virions were used to infect Huh-7 cells (80.2% inhibition; $p \leq 0.0001$) (Figure 4F).

The peptides used are positively charged and amphipathic; these properties may play important roles in their activity against enveloped particles, such as virions of alphaviruses. The viral envelope originates from host cell membranes and contains lipid rafts, sphingolipids, phosphatidylserine and cholesterol, which provide an amphipathic character and negative

charge. Thus, amphipathic cationic peptides can interact with this virus envelope and produce a direct virucidal effect, resulting in interference with virus binding and/or fusion [49]. Similarly, a study from Ahmed and collaborators observed that a cationic peptide can interfere with virus binding by causing aggregation of extracellular virions [50]. Therefore, it is plausible that the peptides used caused inactivation of CHIKV virions by disruption of the viral envelope. Indeed, it was observed by Batista and coworkers that the peptide GA-Hecate was capable of generating disruption of the HCV viral mimetic envelope [20]. Interestingly, however, in this study, the impact of virion treatment clearly depended on the cell line that was infected with peptide-treated particles; for GA-Hecate, no effect of peptide treatment was observed upon infection of Huh-7 cells, while treatment with PSSct1905 or PSSct1910 had no or minimal impact on the ability of CHIKV virions to infect BHK-21 cells. These data argue against the possibility of general destruction (or aggregation) of virions and are more consistent with milder peptide-induced damage. If so, the difference in the expression of receptors on the BHK-21 and Huh-7 cell surfaces and their affinity toward CHIKV could explain this difference. Furthermore, the experimental setup included 1 hour of incubation of cells with inoculum containing both virus particles and peptides. Thus, it is possible that in the presence of peptides, the binding of virions to cells or their entry was also impaired in a cell type-dependent manner. Therefore, the impact of peptides on these steps of the virus infection cycle was subsequently analyzed.

Figure 4. Effect of the peptides-treated CHIKV virions on BHK-21 and Huh-7 cells. Data were analyzed as described for Figure 2. Luminescence signals were measured from three independent experiments, each performed in quadruplicate, and data are presented as the mean ± SD. **(A)** GA-Hecate in BHK-21 cells. **(B)** PSSct1905 in BHK-21 cells. **(C)** PSSct1910 in BHK-21 cells. **(D)** GA-Hecate in Huh-7 cells. **(E)** PSSct1905 in Huh-7 cells. **(F)** PSSct1910 in Huh-7 cells. ******:** $p \leq 0.0001$.

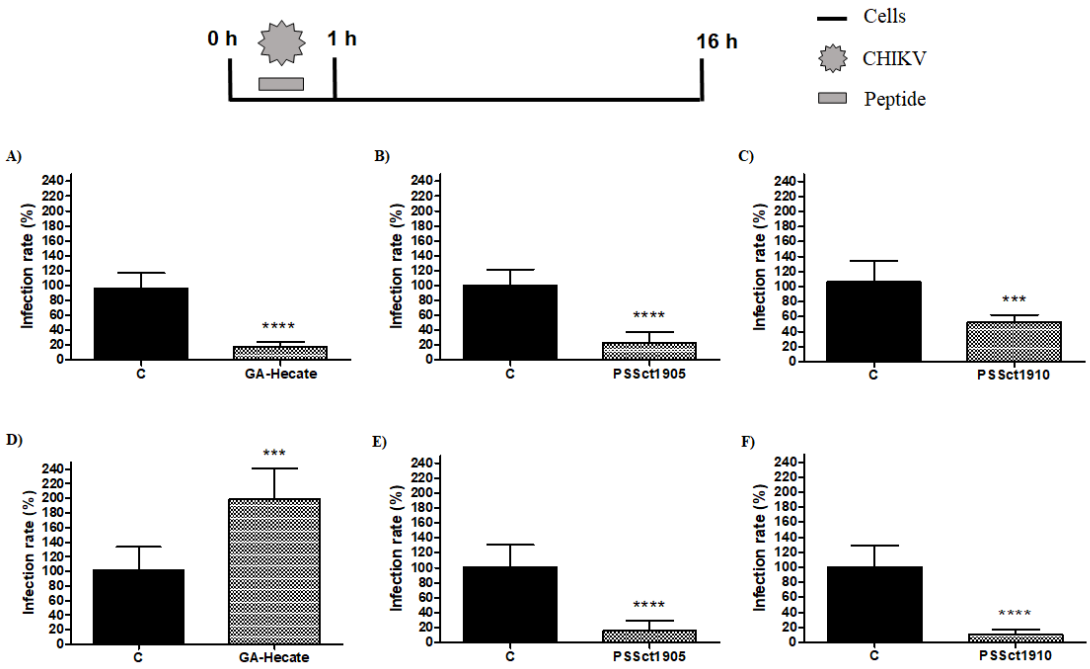


Antiviral activity of the peptides on the CHIKV entry steps

To evaluate the action of the peptides in the early steps of the CHIKV replication cycle, BHK-21 and Huh-7 cells were infected with CHIKV-NLuc at an MOI of 0.1 in the presence of the MNTC of GA-Hecate, PSSct1905, or PSSct1910 for 1 hour, washed with PBS and incubated with peptide-free culture medium. In this experiment, all peptides inhibited CHIKV entry into BHK-21 cells. The effect was strongest for GA-Hecate and PSSct1905, for which 77.2% ($p \leq 0.0001$) and 76.4% ($p \leq 0.0001$) inhibition was observed, respectively (Figures 5A and B). PSSct1910 was somewhat less potent, with an inhibition rate of 54.1% ($p \leq 0.001$) compared to the control (Figure 5C). Curiously, this trend is the same as that observed in the previous experiment (Figures 4A, B and C); the different inhibition rates observed in these experiments can be attributed to the 50-fold higher MOI used in the previous experiment, which makes the assay less sensitive. In Huh-7 cells, GA-Hecate failed to inhibit the early steps of CHIKV infection; instead, significant activation was observed (Figure 5D). At the same time, PSSct1905 and PSSct1910 reduced the efficiency of the entry stages of CHIKV infection and

resulted in 84% ($p \leq 0.0001$) and 89.5% ($p \leq 0.0001$) inhibition, respectively (Figures 5D, E and F). Again, the trend is similar to that observed in the previous experiment (Figures 4D, E and F). Combined with data obtained for BHK-21 cells, these results strongly indicate that antiviral activities observed in the previous experiment were mostly, if not exclusively, due to inhibition of early stages of CHIKV infection. Hence, it is unclear whether the peptides have virucidal effects.

Figure 5. Effect of the peptides on CHIKV entry. Data were analyzed as described for Figure 2. Luminescence signals were measured from three independent experiments, each performed in quadruplicate, and data are presented as the mean ± SD. **(A)** GA-Hecate in BHK-21 cells. **(B)** PSSct1905 in BHK-21 cells. **(C)** PSSct1910 in BHK-21 cells. **(D)** GA-Hecate in Huh-7 cells. **(E)** PSSct1905 in Huh-7 cells. **(F)** PSSct1910 in Huh-7 cells. *****:** $p \leq 0.001$; ******:** $p \leq 0.0001$.



Antiviral effect of the peptides on CHIKV attachment

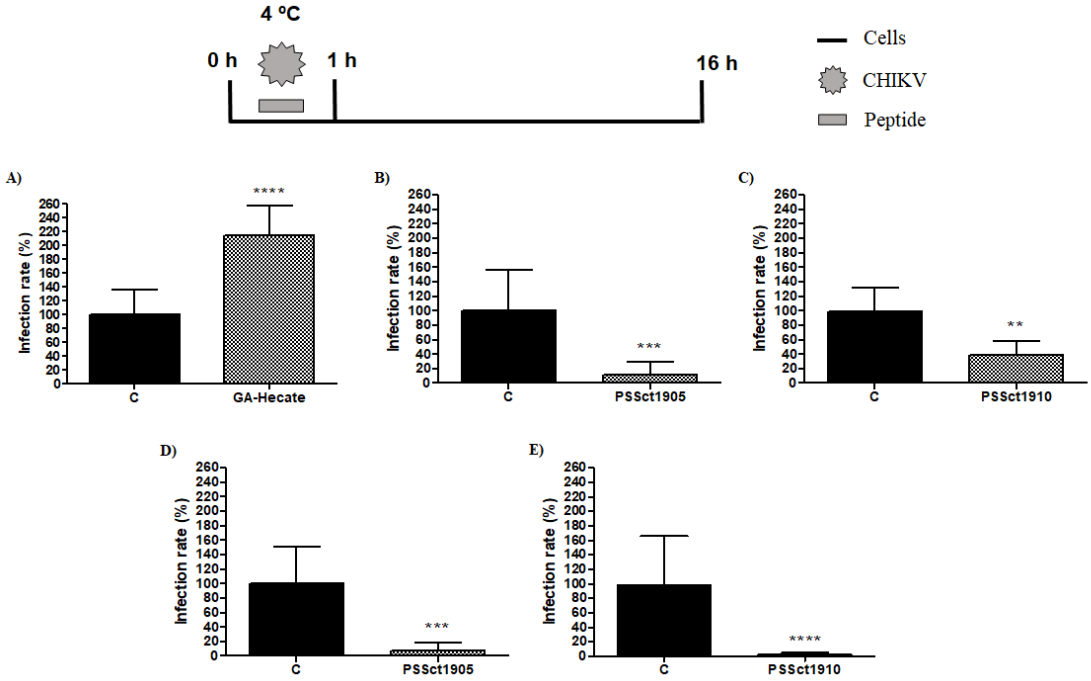
To reveal which processes involved in the entry of CHIKV infection were affected by the peptides, we first investigated their activity on CHIKV attachment. In this experiment, BHK-21 and Huh-7 cells were incubated with peptides at the MNTC and CHIKV-NLuc in amounts corresponding to infection at an MOI of 0.1 at 4 °C. At this temperature, virions interact with cell membrane receptors, but the viral particle is not able to enter the cell [51]. Subsequently, the cells were washed with PBS and incubated with culture medium to continue the viral entry

process. As GA-Hecate was unable to inhibit CHIKV entry into Huh-7 cells (Figure 5D), this combination was excluded from this analysis.

Despite demonstrating an antiviral effect on the entry of CHIKV into BHK-21 cells, the peptide GA-Hecate did not inhibit the viral attachment step (Figure 6A). These data indicate that the peptide GA-Hecate probably inhibits viral entry by interfering with the internalization of CHIKV in BHK-21 cells. In contrast, PSSct1905 and PSSct1910 inhibited the attachment of CHIKV virions to BHK-21 and Huh-7 cells. In BHK-21 cells, treatment with PSSct1910 exhibited less prominent antiviral activity (59.9% inhibition; $p \leq 0.01$) than PSSct1905, which resulted in 89.5% inhibition ($p \leq 0.001$) (Figures 6B and C). In Huh-7 cells, treatment with PSSct1905 or PSSct1910 resulted in very high levels of inhibition with minimal differences between these compounds: 93.5% ($p \leq 0.001$) and 95.3% ($p \leq 0.0001$) inhibition, respectively (Figures 6D and E). Interestingly, these effects were more prominent than those obtained for the inhibition of the complete entry stage (Figures 5B, C, E and F); the difference is probably due to different experimental conditions, such as the low temperature used in the assay that analyzed effect of peptides on the attachment step.

CHIKV entry into cells is mediated by the viral glycoproteins E2 and E1 through several attachment factors — namely, heparan sulfate and phosphatidylserine receptors (TIM1 [52], TIM4 and AXL [53]) — and receptors such as Mxra8 [38]. Cationic peptides can impair virus attachment to host cells by blocking cell receptors and/or by binding to virions [54]. As cited earlier, the peptides of this study have a positive charge similar to the E2 glycoprotein of the CHIKV. This may lead to competition for binding to cellular receptors, resulting in reduced attachment of virions to the cell.

Figure 6. Effect of the peptides on CHIKV attachment. Data were analyzed as described for Figure 2. Luminescence signals were measured from three independent experiments, each performed in quadruplicate, and data are presented as the mean ± SD. **(A)** GA-Hecate in BHK-21 cells. **(B)** PSSct1905 in BHK-21 cells. **(C)** PSSct1910 in BHK-21 cells. **(D)** PSSct1905 in Huh-7 cells. **(E)** PSSct1910 in Huh-7 cells. ****:** $p \leq 0.01$; *****:** $p \leq 0.001$; ******:** $p \leq 0.0001$.



Antiviral action of the peptides on CHIKV internalization

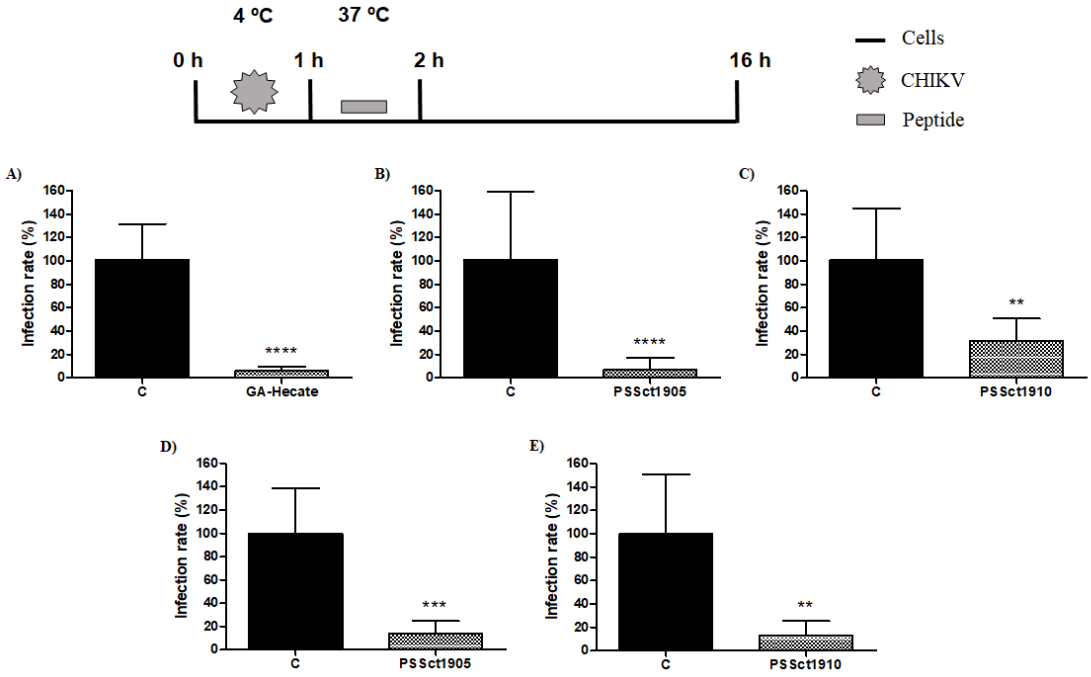
In addition to attachment, the peptides GA-Hecate, PSSct1905 and PSSct1910 were tested for their ability to inhibit CHIKV internalization in BHK-21 and Huh-7 cells. For this analysis, the cells were first incubated with viral particles at 4 °C to allow viral attachment to occur; after this, inoculum was replaced with media containing the peptides at the MNTC, and cells were placed at 37 °C to allow the internalization of the CHIKV particles. After 1 hour, the cells were again washed with PBS and incubated with culture medium to continue the replicative cycle of the virus. As in the previous experiment, the combination of GA-Hecate and Huh-7 cells was excluded from this analysis.

The peptide GA-Hecate, which was unable to inhibit the attachment of CHIKV to BHK-21 cells (Figure 6A), strongly inhibited the internalization of CHIKV particles into BHK-21 cells. The observed inhibition was approximately 95.1% ($p \leq 0.0001$) (Figure 7A). This inhibition is apparently prominent enough to overcome the activation of CHIKV attachment, resulting in inhibition, albeit more modest, of the CHIKV entry step in general (Figure 5A). The viral internalization stage was also inhibited by PSSct1905 and PSSct1910 treatment, resulting in

inhibition of 94.5% ($p \leq 0.0001$) and 69.1% ($p \leq 0.01$), respectively, in BHK-21 cells (Figures 7B and C). High levels of inhibition were also observed in Huh-7 cells, in which treatment with PSSct1905 and PSSct1910 resulted in 85.8% ($p \leq 0.001$) and 87.1% ($p \leq 0.01$) inhibition, respectively (Figures 7D and E). Thus, regardless of the cell type, these analogs inhibited CHIKV entry by interfering with both virus attachment and internalization. Whether these two mechanisms of action can result in additive (or cumulative) effects remains unclear, as the use of different experimental conditions (most notable use of different temperatures) in assays that evaluate entry in general and attachment/internalization stages specifically does not allow direct comparison of data originating from corresponding experiments.

CHIKV enters cells by clathrin-mediated endocytosis. Subsequently, the fusion of the viral and endosomal membranes occurs in a low-pH-dependent manner [55]. Amphipathic and positively charged peptides have been described to inhibit the intracellular trafficking of many pH-dependent enveloped viruses [56]. It is plausible that the peptides used in this study enter the cells together with the virions using endocytic vesicles by binding to cell (and/or virion) surfaces. In endocytic vesicles, they can suppress endosomal acidification, delaying or preventing the activation of pH-triggered viral fusogenic machinery, which is crucial for viral genome release into the cytoplasm. Additionally, it is possible that peptides hinder acidification via their electrostatic contributions to the endosomal lumen, resulting in resistance to the influx of hydrogen ions (driven by H^+ ATPase pumps). Alternatively, peptides may partially act as a buffering agent by sequestering incoming luminal protons. Finally, the peptides might regulate endosomal acidification by directly interacting with the host machinery, which drives this process [57].

Figure 7. Effect of the peptides on CHIKV internalization. Data were analyzed as described for Figure 2. Luminescence signals were measured from three independent experiments, each performed in quadruplicate, and data are presented as the mean ± SD. **(A)** GA-Hecate in BHK-21 cells. **(B)** PSSct1905 in BHK-21 cells. **(C)** PSSct1910 in BHK-21 cells. **(D)** PSSct1905 in Huh-7 cells. **(E)** PSSct1910 in Huh-7 cells. ****:** $p \leq 0.01$; *****:** $p \leq 0.001$; ******:** $p \leq 0.0001$.



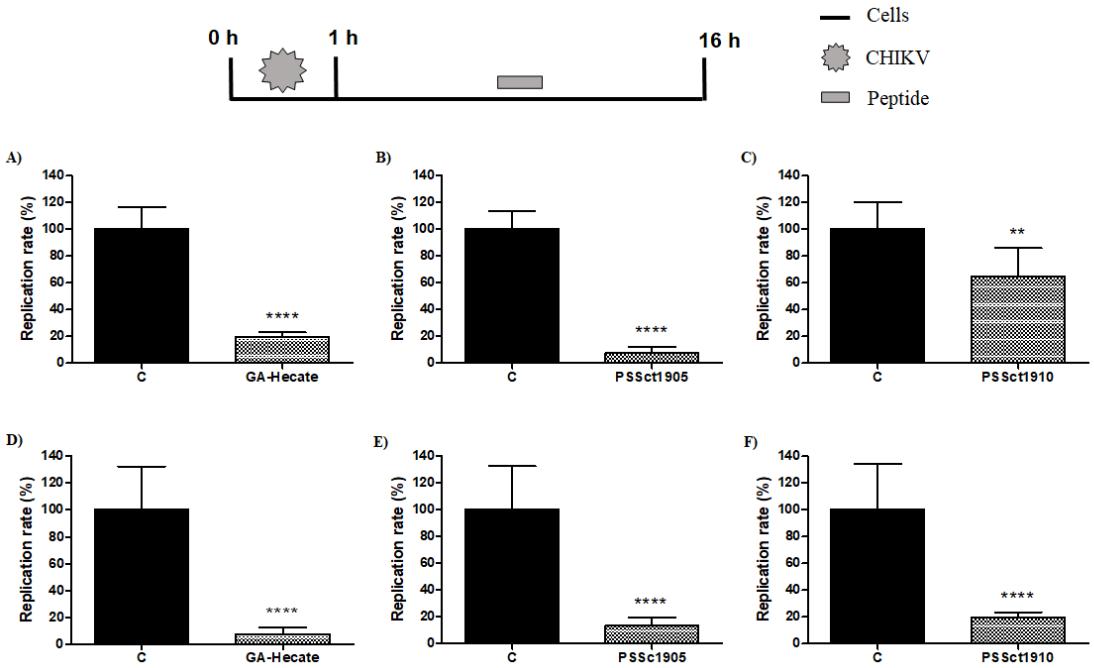
Antiviral activity of the peptides on the postentry steps of CHIKV infection

To evaluate the antiviral activity of the peptides on the postentry stages of the CHIKV replicative cycle, BHK-21 and Huh-7 cells were infected with CHIKV-NLuc at an MOI of 0.1. After 1 hour of incubation, the cells were washed, and media containing peptides at the MNTC was added. In this assay, GA-Hecate inhibited CHIKV infection by 80.7% ($p \leq 0.0001$) and 92.7% ($p \leq 0.0001$) in BHK-21 and Huh-7 cells, respectively (Figures 8A and D). The peptide PSSct1905 caused 92.2% ($p \leq 0.0001$) and 86.8% ($p \leq 0.0001$) reductions in viral replication in BHK-21 and HuH-7 cells, respectively (Figures 8B and E). The lowest activities were observed for the peptide PSSct1910, which reduced viral replication by approximately 35.2% ($p \leq 0.01$) in BHK-21 cells and 80.3% ($p \leq 0.0001$) in Huh-7 cells (Figures 8C and F).

Translation of the alphavirus RNA genome generates one or two nonstructural protein precursors. Cleavage of these precursors results in the formation of the nonstructural proteins nsP1–nsP4 [58,59]. nsP1 demonstrates RNA capping activities and participates in the formation of the viral RNA negative strand; nsP2 triggers blocking of the host transcription process and exhibits RNA helicase, RNA triphosphatase and proteinase activities; nsP3 recruits RNA-

binding proteins that play a role in the formation of CHIKV replication complexes; and nsP4 has RNA-dependent RNA polymerase activity [59,60]. Alphavirus RNA synthesis occurs in replication complexes located in proximity to the plasma membrane [59]. Nonstructural proteins are part of these replication complexes. This complex is responsible for the formation of a full-length negative strand RNA intermediate, which is used as a template for the production of the positive strand of subgenomic and genomic RNAs. There are some cationic peptides that interact with CHIKV nonstructural proteins and block their activities [61-63], impairing viral replication in host cells. Therefore, we suggest that the peptides used in this study may interfere with the synthesis of the CHIKV nonstructural proteins or inhibit their activity. The analysis of potential targets was outside of scope of the current study.

Figure 8. Effect of the peptides on the postentry steps of CHIKV infection. Data were analyzed as described for Figure 2. Luminescence signals were measured from three independent experiments, each performed in quadruplicate, and data are presented as the mean ± SD. **(A)** GA-Hecate in BHK-21 cells. **(B)** PSSct1905 in BHK-21 cells. **(C)** PSSct1910 in BHK-21 cells. **(D)** GA-Hecate in Huh-7 cells. **(E)** PSSct1905 in Huh-7 cells. **(F)** PSSct1910 in Huh-7 cells. ****:** $p \leq 0.01$; ******:** $p \leq 0.0001$.



CONCLUSION

Our study reports the first inhibitory effects of the peptide GA-Hecate and its analogs PSSct1905 and PSSct1910 against CHIKV infection in BHK-21 and Huh-7 cells. Antiviral activities were observed when the peptides were used as prophylactic treatment and/or were

administered to CHIKV infected cells at early and later stages of infection. These peptides exhibit potentials for further development into anti-CHIKV drugs which could help combat this mosquito-borne viral infection.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary Material

The synthetic peptide GA-Hecate and its analogs inhibit multiple steps of the CHIKV infection cycle *in vitro*

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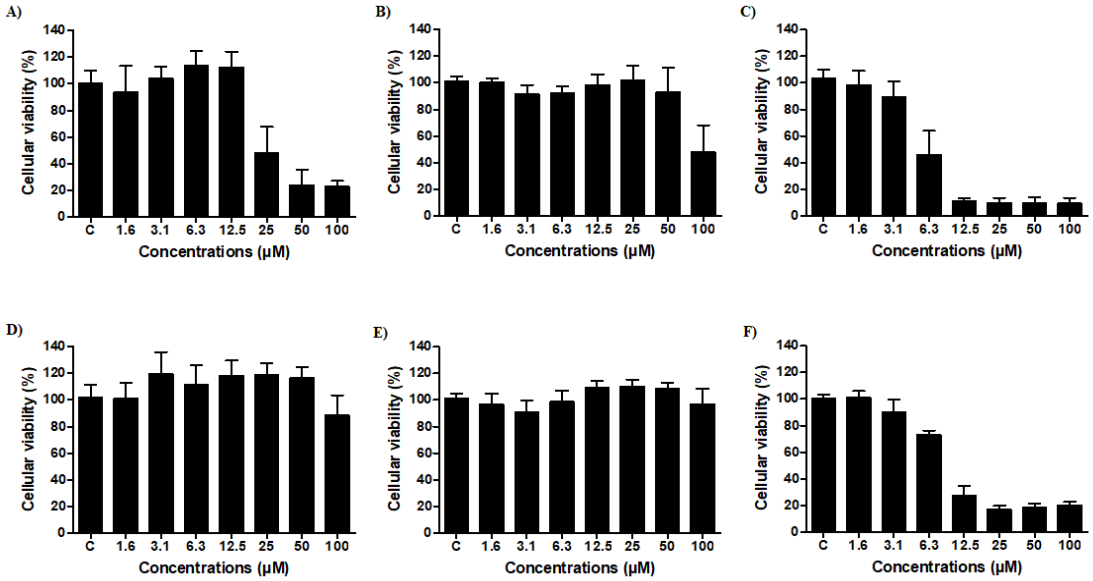
Figure S1. Cytotoxicity of the peptides in BHK-21 and Huh-7 cells. Peptides were incubated at different concentrations (1.6 to 100 µM) in BHK-21 and Huh-7 cells. After 24 hours, the cell viability was analyzed using MTT method. The percentage values are expressed in relation to the results obtained in cells treated with the control (**C**, sterile water). **(A)** GA-Hecate in BHK-21 cells. **(B)** PSSct1905 in BHK-21 cells. **(C)** PSSct1910 in BHK-21 cells. **(D)** GA-Hecate in Huh-7 cells. **(E)** PSSct1905 in Huh-7 cells. **(F)** PSSct1910 in Huh-7 cells.



Capítulo II

*Artigo Científico II**

***Nota:** O próximo artigo científico é resultado de duas teses de doutorado distintas, nas quais os dados foram combinados para uma única publicação. Porém, a parte desenvolvida no presente trabalho corresponde aos resultados obtidos para o vírus Chikungunya.

Viruses

The Dimeric Peptide $(KKYRYHLKPF)_2K$ Shows Broad-Spectrum Antiviral Activity by Inhibiting Different Steps of Chikungunya and Zika Virus Infection

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Abstract

Chikungunya virus (CHIKV) and Zika virus (ZIKV) are important disease-causing agents worldwide. Currently, there are no antiviral drugs or vaccines approved to treat these viruses. However, peptides have shown great potential for new drug development. A recent study described (p-BthTX-I)$_2$K [(KKYRYHLKPF)$_2$K], a peptide derived from the Bothropstoxin-I toxin in the venom of the *Bothrops jararacussu* snake, showed antiviral activity against SARS-CoV-2. In this study, we assessed the activity of this peptide against CHIKV and ZIKV and its antiviral action in the different stages of the viral replication cycle *in vitro*. We observed that (p-BthTX-I)$_2$K impaired CHIKV infection by interfering with the early steps of the viral replication cycle, reducing CHIKV entry into BHK-21 cells, specifically by reducing both the attachment and internalization steps. (p-BthTX-I)$_2$K also inhibited the ZIKV replicative cycle in Vero cells. The peptide protected the cells against ZIKV infection and decreased the levels of the viral RNA and the NS3 protein of this virus at viral postentry steps. In conclusion, this study highlights the potential of the (p-BthTX-I)$_2$K peptide to be a novel broad-spectrum antiviral candidate that targets different steps of the replication cycle of both CHIKV and ZIKV.

Keywords: CHIKV. ZIKV. Antiviral. Peptide.

Introduction

Arboviruses constitute a group of viruses that are transmitted between vertebrate hosts by hematophagous arthropod vectors, such as ticks and mosquitoes [1]. To date, more than 500 species of arboviruses belonging to 14 different families have been reported, with more than 100 species identified as human or animal pathogens [2]. Arboviruses that cause disease in humans or animals belong to four main families: *Peribunyaviridae*, *Flaviviridae*, *Togaviridae*, and *Sedoreoviridae* [2,3]. Temperature, climate, and vegetation are ecological parameters that interfere with both vectors and host distribution [4]. Brazil is a large and tropical country with many characteristics favorable to arbovirus survival, such as climate, vegetation, and biodiversity [5]. Zika virus (ZIKV) and Chikungunya virus (CHIKV) are among the main arboviruses causing infection in Brazil [6,7].

CHIKV is an enveloped virus with a single-stranded, positive-sense RNA genome that belongs to the *Togaviridae* family and *Alphavirus* genus. This virus has a worldwide distribution and is considered a serious public health problem. According to the Pan American Health Organization (PAHO), more than 250,000 cases of CHIKV infection were reported in 12 countries and territories of the Americas in 2022. Among these countries, Brazil reported the highest incidence, with 98.8% of the cases [8]. CHIKV infection triggers symptoms in 72 to 95% of patients [9], and symptoms associated with pain and swelling, particularly in the wrists, hands, ankles, and feet, can persist for years [10]. To date, no specific antiviral therapy or licensed vaccine is available for CHIKV infection prevention or treatment, and only palliative care is recommended to alleviate the symptoms of disease [9,11].

ZIKV is an enveloped positive-sense single-stranded RNA virus. It belongs to the *Flaviviridae* family in genus *Flavivirus* [12]. ZIKV caused an epidemic in the Americas in approximately 2015, and during this period, infection in pregnant women was related to a set of neurological alterations in their fetuses named congenital Zika syndrome (CZS) [13-15]. ZIKV infection can also cause Guillain–Barré syndrome and other neurological complications in adults [16]. Thus, although the majority of ZIKV infection is asymptomatic, this virus is an important public health concern, especially because of CZS [15]. In 2022, more than 31,400 ZIKV infection cases were reported in the Region of the Americas according to the PAHO. Most of these cases were reported in Brazil, where 92.6% of the cases were reported [8]. Similar to the case of CHIKV, there are no vaccines or antivirals approved against ZIKV [17]. Therefore, there is a significant need for treatments or vaccines that are safe and effective in reducing viral spread and limiting the disease burden caused by CHIKV or ZIKV.

In recent decades, the discovery of the therapeutic potential of several peptides with different biological targets has increased the interest of the scientific community in this class of compounds [18,19]. Peptides have cellular biocompatibility, demonstrating high affinity and specificity, which makes them potential drug candidates [18-20]. Antimicrobial peptides (AMPs) are derived from natural or synthetic sources. Among the synthetic peptides there are analogs generated on the basis of natural peptide structures [18]. The peptide (p-BthTX-I)$_2$ [(KKYRYHLKPFCKK)$_2$], derived from the Bothropstoxin-I (BthTX-I) toxin in *Bothrops jararacussu* snake venom, has been shown to carry antibacterial activity against Gram-positive and Gram-negative bacteria [21]. Santos-Filho and coworkers demonstrated that the removal of four lysine residues located in the C-terminal region of the peptide (p-BthTX-I)$_2$ [des-Lys^{12}, Lys^{13}-(p-BthTX-I)$_2$] [(KKYRYHLKPFC)$_2$] did not lead to a lack of antibacterial activity [22]. Another work reported by the same group indicated that the absence of Lys^{12} and Lys^{13}, the removal of two cysteine residues, and the insertion of a lysine residue at a branch point in the C-terminal region [des-Cys^{11}, Lys^{12}, Lys^{13}-(p-BthTX-I)$_2$K – (KKYRYHLKPF)$_2$K] [(p-BthTX-I)$_2$K peptide] resulted in greater antibacterial activity than the wild-type peptide (p-BthTX-I)$_2$ [23]. The synthetic peptide also showed antiviral activity against severe acute respiratory syndrome virus 2 (SARS-CoV-2), an enveloped virus that belongs to the *Coronaviridae* family [24].

This study aimed to evaluate the anti-CHIKV and anti-ZIKV activity of the (p-BthTX-I)$_2$K peptide and investigate its effects on different steps of the viral infection cycle. These data constitute the first report of the antiviral activity of the (p-BthTX-I)$_2$K peptide against *Alphavirus* and *Flavivirus* infections.

Materials and Methods

Peptide

The (p-BthTX-I)$_2$K peptide [(KKYRYHLKPF)$_2$K] (molecular weight = 2869.45 g/mol) was synthesized according to the method reported by Santos-Filho and coworkers [23]. In brief, the peptide was prepared on a TRIBUTE-UV automatic synthesizer (Protein Technologies, Tucson, AZ, USA) via solid phase peptide synthesis (SPPS) using the standard Fmoc protocol (9-fluorenylmethyloxycarbonyl) on a Rink-MBHA resin. To obtain the dimer, Fmoc-Lys(Fmoc)-OH was added to the C-terminus as a branch point, allowing for the growth of peptide chains from the α-amino and ε-amino groups. The peptide identity was confirmed by

electrospray ionization mass spectrometry. The purity of the (p-BthTX-I)$_2$K peptide was higher than 95%.

Cells

Baby hamster kidney fibroblast cells (BHK-21; ATCC CCL-10) and African green monkey kidney cells (Vero; ATCC CCL-81) were cultured in Dulbecco's modified Eagle's medium (DMEM, Cultilab, Campinas, SP, Brazil) supplemented with 10% fetal bovine serum (FBS, Gibco—Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin (10,000 IU/mL)/streptomycin (10 mg/mL) (P/S) (Cultilab, Campinas, SP, Brazil). The cells were maintained in a humidified incubator at 37 °C in 5% CO_2.

C6/36 cells (ATCC CRL-1660) from *Aedes albopictus* mosquitoes were cultured in Leibovitz L-15 medium (Cultilab, Campinas, SP, Brazil) supplemented with 10% FBS (Gibco—Thermo Fisher Scientific, Waltham, MA, USA) and 1% P/S (Cultilab, Campinas, SP, Brazil). These cells were maintained in an incubator at 28 °C.

Viruses

The recombinant CHIKV-NLuc used here was based on the CHIKV isolate LR2006OPY1 (East/Central/South African genotype). The infectious cDNA plasmid of this virus carries the human cytomegalovirus (CMV) promoter upstream of the sequence corresponding to the viral genome; the sequence of the reporter gene that encodes the NanoLuciferase (NLuc) protein was inserted into the region encoding the nsP3 protein of CHIKV [25]. ZIKV^{BR} – BeH815744, isolated from a febrile patient in Paraíba state, Brazil, was used for the experiments with ZIKV [26].

Viral Stock Preparation

To produce infectious viral particles of CHIKV-NLuc, BHK-21 cells seeded in 24-well culture plates (TPP, Trasadingen, Switzerland) (1×10^5 cells/well) were transfected with 1 µg of CMV-CHIKV-NLuc plasmid using Lipofectamine 2000 ((Thermo Fisher Scientific, Waltham, MA, USA) and OPTI-MEM (reduced serum medium) (Gibco—Thermo Fisher Scientific, Waltham, MA, USA) following a previously described protocol with modifications [27]. Seventy-two hours post-transfection, the supernatant was collected and stored at -80 °C.

For preparing ZIKV stock, C6/36 cells were infected with virus and incubated for 48–96 hours until a cytopathic effect was observed. The supernatant of the infected cells was collected, filtered, and stored at –80 °C.

Viral stock titers were prepared via the plaque-forming method according to the procedure used by Santos and coworkers with modifications [27]. BHK-21 cells were used for CHIKV infection, while Vero cells were used for ZIKV infection. These cells were cultured in 24-well culture plates (TPP, Trasadingen, Switzerland) (1×10^5 cells/well) and infected with 10-fold serial dilutions of the respective virus for 1 hour at 37 °C. Then, the viral inoculum was removed, and a semisolid medium consisting of DMEM (Cultilab, Campinas, SP, Brazil) and 2% carboxymethylcellulose (CMC) (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 1% FBS ((Gibco—Thermo Fisher Scientific, Waltham, MA, USA) and 1% P/S (Cultilab, Campinas, SP, Brazil) was immediately added. After incubation of the plate for 48 hours (for CHIKV) and 96 hours (for ZIKV), the cells were fixed with 10% formaldehyde (Merck, Darmstadt, Germany) and stained with 1% crystal violet (Merck, Darmstadt, Germany). The viral plaques were counted to determine the infectivity titer of each virus, which was expressed in plaque-forming units per milliliter (PFU/mL).

Cytotoxicity Analysis

The toxicity of the (p-BthTX-I)$_2$K peptide in BHK-21 and Vero cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described [28] with modifications. The cells were seeded in 96-well culture plates (TPP, Trasadingen, Switzerland) (5×10^3 cells/well) for 24 hours. Then, the cells were incubated with the (p-BthTX-I)$_2$K peptide at concentrations of 1.6, 3.1, 6.3, 12.5, 25, 50, and 100 µM. After 24 hours (BHK-21) or 48 hours (Vero), the medium containing the peptide was aspirated, and 100 µL of MTT (Sigma–Aldrich, St. Louis, MO, USA) diluted in DMEM (Cultilab, Campinas, SP, Brazil) (final concentration: 1 mg/mL) was added to the cells. After 30 minutes of incubation at 37 °C, the medium containing MTT (Sigma–Aldrich, St. Louis, MO, USA) was removed, and 100 µL of dimethylsulfoxide (DMSO) (Synth, Diadema, SP, Brazil) was added to each well of the plate, which was rotated at 200 rpm for 5 minutes. The absorbance was measured at a wavelength of 572 nm with a plate reader (FLUOstar Omega/BMG LABTECH, Ortenberg, Germany).

Analysis of the Activity of the CHIKV-NLuc-Encoded Reporter

The antiviral activity of the (p-BthTX-I)$_2$K peptide against CHIKV-NLuc was determined via the measurement of the activity of the virus-encoded NLuc reporter. After incubation, the supernatant was removed, the cells were washed with PBS (phosphate-buffered saline) solution, and 30 µL of Renilla luciferase assay lysis buffer (Promega, Madison, WI, USA) was

immediately added to each well. After 30 minutes, the plates containing the cell lysates were placed in a plate reader (FLUOstar Omega/BMG LABTECH, Ortenberg, Germany), where 50 µL of the substrate for Renilla Luciferase (Renilla Luciferase assay reagent, Promega, Madison, WI, USA) was automatically injected into the wells. The light intensity was read, and the values obtained were expressed as a percentage of expression compared with that of the vehicle control (VC) (sterile water).

ZIKV RNA Yield Inhibition Assay Using Reverse-Transcription–Quantitative RT–PCR (qRT–PCR)

Briefly, (p-BthTX-I)$_2$K antiviral activity was evaluated based on the detection of ZIKV RNA copy numbers via qRT–PCR assay. Supernatants from each well were collected at the end of every experiment, and total RNA was extracted using TRIzol reagent (Invitrogen, Waltham, MA, USA) following the manufacturer's instructions. Viral load was then quantified using two-step qRT–PCR. The cDNA was synthesized with a high-capacity cDNA archive kit (Applied Biosystems, Waltham, MA, USA) according to the guidelines of the manufacturer. Quantification of the synthesized cDNA was then carried out using a QuantStudio™ 12 K Flex Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) utilizing ZIKV 1086, ZIKV 1162c, and 1107-FAM primers [29] and TaqMan Universal PCR Master Mix, No AmpErase® UNG (Thermo Fisher Scientific, Waltham, MA, USA) and 1 µL of cDNA/reaction. The thermal cycler parameters were initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The cycle threshold (C_t) was analyzed, and the ZIKV RNA copy numbers of each sample were determined based on the standard curve and expressed on a $\log_{10}$ scale. For the postentry assay, total RNA was isolated from infected cells. To analyze the abundance of ZIKV RNA in these samples, the amounts were normalized to the mRNA level of β-actin; the abundance of the latter was analyzed using primers (F: 5' CAGCACAATGAAGATCAAGAT; R: 5' – CTAGAAGCATTTGCGGTGGAC, 5' – [6FAM]ACCTTCCAGCAGATGTGGATC[BHQ1]), following the ΔCt method with QuantStudio™ 12K Flex software v1.4.

Western Blot Analysis

To evaluate the NS3 protein of ZIKV expression in the postentry assay was performed a western blot assay. The cells from the postentry assay were collected and the cell pellet was lysed with the CelLytic M buffer (Sigma–Aldrich, St. Louis, MO, USA) containing the Protease Inhibitor

Cocktail (Promega, Madison, WI, USA). The extracted proteins were quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) to ensure equal loading in SDS-PAGE 10%. After protein denaturation, the proteins were separated in an electrophoresis system and transferred onto a polyvinylidene fluoride (PVDF) membrane (Merck, Darmstadt, Germany). After blotting, the transferred PVDF membrane was blocked for 1h at room temperature (RT) with TBS buffer containing 5% non-fat dry milk and 0.05% Tween 20. The PVDF membrane was incubated overnight at 4°C with rabbit polyclonal anti-ZIKV NS3 antibody (1:2000, diluted in blocking buffer), kindly provided by Dr. Andres Merits, from the University of Tartu, Estonia, or rabbit anti-GAPDH monoclonal antibody (1:1000; #14C10; Cell Signaling, Danvers, MA, USA). The PVDF membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000; #31460; Thermo Scientific™, Waltham, MA, USA) for 1h at RT. Between each reaction step, washing was performed with a TBS buffer containing 0.05% Tween 20. Finally, the target proteins were detected by ECL substrate with a ChemiDoc Imager (BioRad, Hercules, CA, USA). The band intensity of NS3 viral protein was calculated using Image Lab software (BioRad, Hercules, CA, USA) and normalized to GAPDH.

Primary Antiviral Analysis of (p-BthTX-I)$_2$K Activity against CHIKV and ZIKV Infection

The antiviral activity of the (p-BthTX-I)$_2$K peptide against CHIKV and ZIKV infection was analyzed according to a method previously described by Oliveira and coworkers [30] and Silva and collaborators [31] with modifications, respectively. For CHIKV screening, 1×10^4 BHK-21 cells were seeded in the wells of 96-well white culture plates (Greiner Bio-one, Americana, SP, Brazil). For ZIKV screening, 2×10^4 Vero cells were seeded in the wells of a 24-well culture plate (TPP, Trasadingen, Switzerland). Twenty-four hours later, CHIKV-NLuc or ZIKV^{BR} at a multiplicity of infection (MOI) of 0.1 and the (p-BthTX-I)$_2$K peptide at the maximum nontoxic concentration (MNTC), previously established with an MTT assay, were simultaneously added to the cells. The plate was then incubated at 37 °C for 16 hours (CHIKV) or 48 hours (ZIKV). The inhibitory effect of (p-BthTX-I)$_2$K on CHIKV was evaluated via the measurement of the NLuc activity and of ZIKV via qRT–PCR as described above.

Antiviral Dose–Response Assay

The 50% effective concentration (EC_{50}) for CHIKV and ZIKV inhibition was determined by the antiviral dose-response assay, as previously described [30,31] with modifications. For CHIKV,

1×10^4 BHK-21 cells were seeded in the wells of 96-well white culture plates (Greiner Bio-one, Americana, SP, Brazil) and for ZIKV, 2×10^4 Vero cells/well were seeded in a 24-well plate (TPP, Trasadingen, Switzerland), both 24 hours before the treatment and infection. CHIKV-NLuc at an MOI of 0.1 and the (p-BthTX-I)2K peptide at a range of concentrations of 3.1, 6.3, 12.5, 25, and 50 µM were simultaneously added to the cells and incubated for 16 hours at 37º C. To ZIKV, the procedure was similar to the CHIKV, but the concentrations were 6.3, 12.5, 25, 50, and 100 µM and the ZIKV MOI 0.1 and the peptide concentrations were incubated for 48 hours. The inhibitory effect of (p-BthTX-I)2K on the CHIKV infection cycle was evaluated by measuring NLuc activity and on ZIKV infection by qRT-PCR. The EC_{50} values were calculated by non-linear regression of the dose-response curves (Log [peptide] × response).

Analysis of the Protective Effect of (p-BthTX-I)$_2$K against CHIKV and ZIKV Infection

The protective action of the (p-BthTX-I)$_2$K peptide against CHIKV and ZIKV infection was evaluated according to the method described by Oliveira and coworkers [30] and Carneiro and collaborators [32] with modifications, respectively. The cells were seeded and incubated as described above were then treated with (p-BthTX-I)$_2$K at the MNTC. After 1 hour of incubation at 37 °C, the supernatant was aspirated, the wells were washed twice with PBS, and CHIKV-NLuc or ZIKV (MOI 0.1) was added to the cells. The plate was incubated at 37 °C for 16 hours (CHIKV) or 48 hours (ZIKV). The inhibitory effect of (p-BthTX-I)$_2$K on CHIKV infection was evaluated via the measurement of the NLuc activity and of ZIKV via qRT–PCR as described above.

Investigation into the Virucidal Effect of (p-BthTX-I)$_2$K

The investigation of the virucidal action of (p-BthTX-I)$_2$K against CHIKV was performed following previously described methods [27,30] with modifications. For ZIKV, this assay was adapted based on a protocol described by Carneiro and collaborators [32]. For CHIKV, 1×10^4 BHK-21 cells were seeded in the wells of 96-well white culture plates (Greiner Bio-one, Americana, SP, Brazil), and for ZIKV, 2×10^4 Vero cells were seeded in the wells of 24-well culture plates (TPP, Trasadingen, Switzerland). The virions of CHIKV-NLuc at an amount to achieve infection with an MOI of 5 or ZIKV at an amount to achieve infection with an MOI of 0.1 were incubated with the (p-BthTX-I)$_2$K peptide at the MNTC at 37 °C (CHIKV) or room temperature (ZIKV) for 1 hour. Then, the viral inoculum was added to cells. After 1 hour of incubation, the supernatant was removed, the cells were washed twice with PBS, and DMEM

(Cultilab, Campinas, SP, BR) supplemented with 2% FBS (Gibco—Thermo Fisher Scientific, Waltham, MA, USA) was added to each well. The cells were incubated at 37 °C for the specific period previously determined for each virus. Sixteen hours post-infection (h.p.i.) CHIKV replication was quantified by measuring NLuc activity. For ZIKV, the viral RNA in the supernatant of the cells was quantified by qRT–PCR 48 h.p.i.

Evaluation of the Antiviral Effect of (p-BthTX-I)$_2$K on CHIKV and ZIKV Entry into Cells

The antiviral activity of the (p-BthTX-I)$_2$K peptide mediated by blocking viral entry into the cells was analyzed according to previously described methods [27,30,33] with modifications. BHK-21 cells (for CHIKV) and Vero cells (for ZIKV) were plated as described above. After 24 hours of culture, the cells were infected with CHIKV-NLuc at an MOI of 0.1 or ZIKV^{BR} at an MOI of 0.1 in the presence of the (p-BthTX-I)$_2$K peptide at the MNTC. After 1 hour of incubation at 37 °C, the supernatant was aspirated, the wells were washed twice with PBS, and DMEM (Cultilab, Campinas, SP, Brazil) with 2% FBS (Gibco—Thermo Fisher Scientific, Waltham, MA, USA) was added to the cells. The plate was incubated at 37 °C for 16 h.p.i (CHIKV) or 48 h.p.i (ZIKV). (p-BthTX-I)$_2$K inhibitory effects on CHIKV were evaluated via the measurement of the NLuc activity, and the effect on ZIKV was evaluated via qRT–PCR as described above.

Analysis of the Impact of (p-BthTX-I)$_2$K on CHIKV Attachment to Cells

The antiviral effect of the (p-BthTX-I)$_2$K peptide on CHIKV attachment to cell receptors was evaluated according to previously described methods [27,30,34] with modifications. In brief, 1×10^4 BHK-21 cells were plated in the wells of 96-well white culture plates (Greiner Bio-one, Americana, SP, Brazil) and cultured for 24 hours. Then, the plate was incubated at 4 °C for 15 minutes, and CHIKV-NLuc (MOI 0.1) and the (p-BthTX-I)$_2$K peptide at the MNTC were simultaneously added. The plate was again incubated at 4 °C. At this temperature, viral particles interact with cell receptors, but the virus cannot enter the cells [27]. After 1 hour, the supernatant was removed, the cells were washed twice with PBS, and DMEM (Cultilab, Campinas, SP, BR) supplemented with 2% FBS (Gibco—Thermo Fisher Scientific, Waltham, MA, USA) was added. The cells were incubated at 37 °C for 16 h.p.i. The inhibitory effect of (p-BthTX-I)$_2$K on CHIKV attachment to host cells was evaluated via the measurement of the NLuc activity.

Evaluation of the Antiviral Action of (p-BthTX-I)$_2$K in CHIKV Internalization into Cells

The antiviral action of the (p-BthTX-I)$_2$K peptide on CHIKV internalization into cells was analyzed according to previously described methods [34] with modifications. BHK-21 cells (1×10^4 cells/well of 96-well white culture plate) were seeded 24 hours before the experiment; then, the plate was incubated at 4 °C for 15 minutes. The cells were infected with CHIKV-NLuc (MOI 0.1). After 1 hour of incubation at 4 °C, the supernatant was aspirated, and the wells were washed twice with PBS and supplemented with culture medium containing the (p-BthTX-I)$_2$K peptide at the MNTC. After 1 hour of incubation at 37 °C, the supernatant was removed, the wells were again washed twice with PBS, and DMEM (Cultilab, Campinas, SP, Brazil) with 2% FBS (Gibco—Thermo Fisher Scientific, Waltham, MA, USA) was added to the cells. The plate was incubated at 37 °C for 16 hours h.p.i., and CHIKV inhibition was evaluated via measurement of the NLuc activity.

Analysis of the Antiviral Effect of (p-BthTX-I)$_2$K on the Postentry Stages of CHIKV and ZIKV Infection

The antiviral activity of the (p-BthTX-I)$_2$K peptide at the postentry stages of viral infection was evaluated according to previously described methods [27,30] with modifications. BHK-21 and Vero cells were plated and cultured as described above and incubated with CHIKV-NLuc or ZIKV^{BR} (MOI 0.1) for 1 hour at 37 °C. Then, the supernatant was aspirated, the cells were washed twice with PBS, and the (p-BthTX-I)$_2$K peptide at the MNTC was added to each well. The cells were incubated at 37 °C for the period previously determined for each virus. Sixteen h.p.i. CHIKV replication was quantified via the measurement of the NLuc activity. For ZIKV^{BR}, the viral RNA in the supernatant of the cells as well as intracellular viral RNA was quantified via qRT–PCR 48 h.p.i.

Statistical Analysis

The cytotoxicity and antiviral activity experiments were performed via three independent assays. For CHIKV, each biological replicate was performed in three (cytotoxicity) and four (antiviral activity) technical replicates; for the ZIKV assays, three (cytotoxicity) and two (antiviral activity) technical replicates were established. The statistical significance of the cytotoxic and antiviral effects of the peptide was determined using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). The statistical analyses were performed by paired Student's t test for parametric data and Mann–Whitney test for nonparametric results.

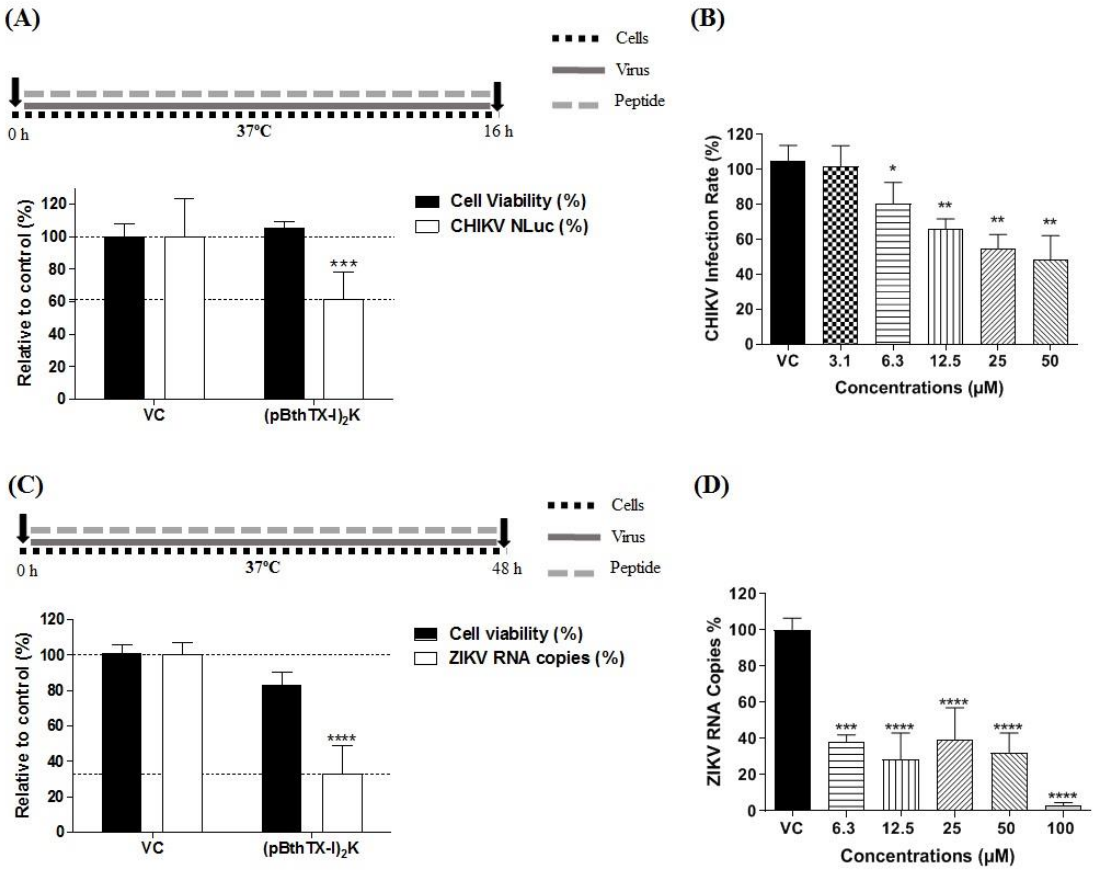
A p value < 0.05 was considered to be statistically significant. The data obtained were normalized to the VC and expressed as percentages.

Results

The (p-BthTX-I)$_2$K Peptide Shows Antiviral Activity against CHIKV and ZIKV^{BR}

The MNTC of the (p-BthTX-I)$_2$K peptide in BHK-21 and Vero cells was 12.5 µM and 25 µM, respectively (Figure S1A and S1B, Supplementary Material). When applied at the MNTC, the (p-BthTX-I)$_2$K peptide reduced CHIKV replication by 38.3% ($p \leq 0.001$) compared to the effect of the VC (Figure 1A); ZIKV^{BR} RNA levels were decreased by approximately 67.3% ($p \leq 0.0001$) compared to those in the VC group (Figure 1C). In addition to this, the (p-BthTX-I)$_2$K peptide impaired the CHIKV (Figure 1B) and ZIKV^{BR} (Figure 1D) infection in a dose-dependent manner, with EC_{50} values of 15.84 µM and 6.5 µM, respectively. Thus, the peptide significantly inhibited the infection of these two arboviruses, warranting subsequent analysis of the effects at different stages of the respective CHIKV and ZIKV infection cycle.

Figure 1. (p-BthTX-I)$_2$K inhibits CHIKV and ZIKV^{BR} infection. **(A)** BHK-21 cells were incubated with (p-BthTX-I)$_2$K at the MNTC (12.5 µM) or **(B)** at concentrations of 3.1, 6.3, 12.5, 25 and 50 µM, and infected with CHIKV-NLuc at an MOI of 0.1. CHIKV replication was analyzed via the measurement of the NLuc activity 16 h.p.i. Mean values ± standard deviations (SDs) were calculated with data obtained from a minimum of three independent experiments that had each been performed in quadruplicate. **(C)** Vero cells were incubated with (p-BthTX-I)$_2$K at 25 µM (the MCNT) or **(D)** at concentrations of 6.3, 12.5, 25, 50 and 100 µM and infected with ZIKV^{BR} at an MOI of 0.1. The RNA levels of ZIKV^{BR} in the cell culture supernatant 48 h.p.i. were quantified by qRT–PCR. Mean values ±SDs represent data from three independent experiments, each of which was performed in duplicate. ***:** $p \leq 0.05$; ****:** $p \leq 0.01$; *****:** $p \leq 0.001$; ******:** $p \leq 0.0001$. **VC:** vehicle control (sterile water). A schematic representation of the respective experiment is shown above each graph.

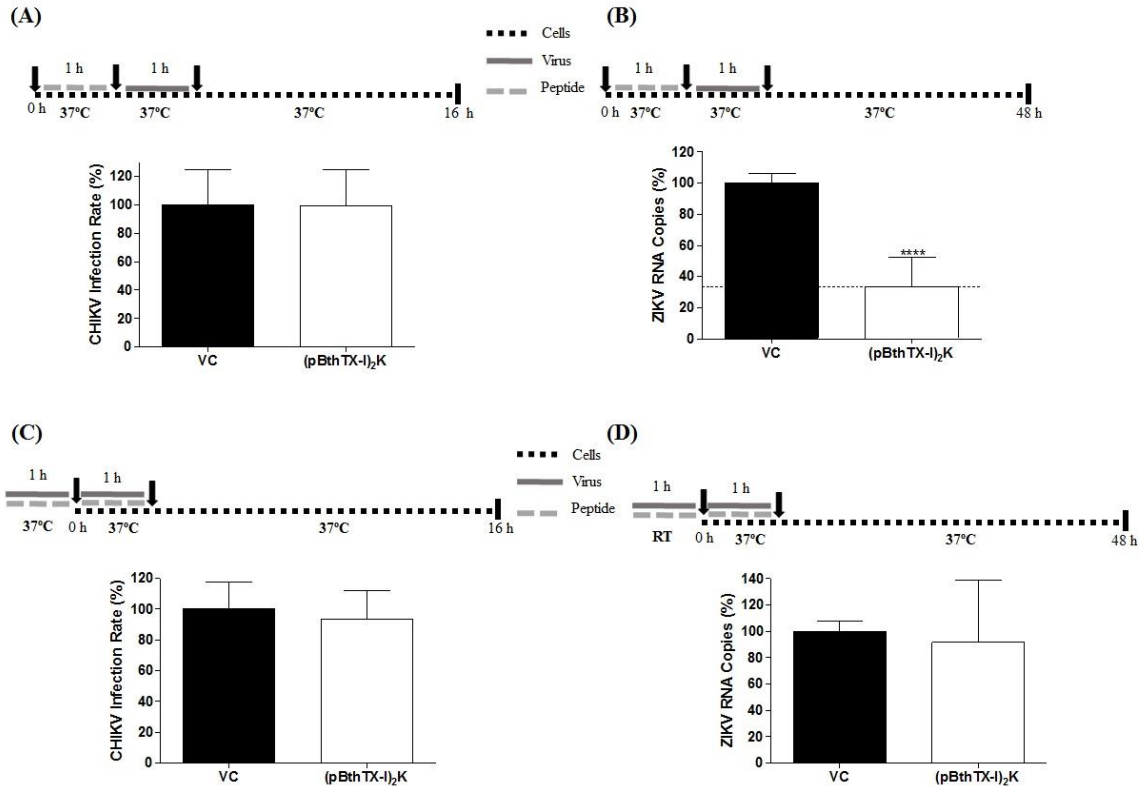


Prophylactic Effect of (p-BthTX-I)$_2$K on CHIKV and ZIKV^{BR} Infection

To assess the protective effect of (p-BthTX-I)$_2$K against CHIKV and ZIKV infection, cells were pretreated with the peptide at the MNTC. After 1 hour of incubation, the cells were washed with PBS to remove the peptide and then were infected with CHIKV-NLuc or ZIKV^{BR}.

Pretreatment with the (p-BthTX-I)$_2$K peptide did not protect BHK-21 cells against CHIKV infection (Figure 2A). However, pretreatment with this peptide protected Vero cells against ZIKV, as evidenced by the significant reduction in the ZIKV RNA levels, of 66.5% ($p \leq$ 0.0001), in treated cultures compared to VC-treated cultures (Figure 2B).

Figure 2. Prophylactic and virucidal activity of the (p-BthTX-I)$_2$K peptide. **(A)** BHK-21 cells pretreated with the peptide at 12.5 µM showed no protection against CHIKV-NLuc infection (MOI of 0.1). **(B)** Treatment of Vero cells with peptide at 25 µM protected cells against infection of ZIKV^{BR} (MOI of 0.1). **(C)** The peptide treatment at 12.5 µM exerted no impact on CHIKV virions. **(D)** The peptide at 25 µM exerted no impact on the ZIKV^{BR} virions. CHIKV replication was analyzed via measurement of the NLuc activity 16 h.p.i. The RNA levels of ZIKV^{BR} in the cell culture supernatant were quantified by qRT–PCR 48 h.p.i. Mean values ± SDs represent data from a minimum of three independent experiments that were each performed in quadruplicate for the CHIKV experiments and in duplicate for the ZIKV experiments. ******:** $p \leq 0.0001$. **VC:** vehicle control (sterile water). A schematic representation of the respective experiment is shown above graph.



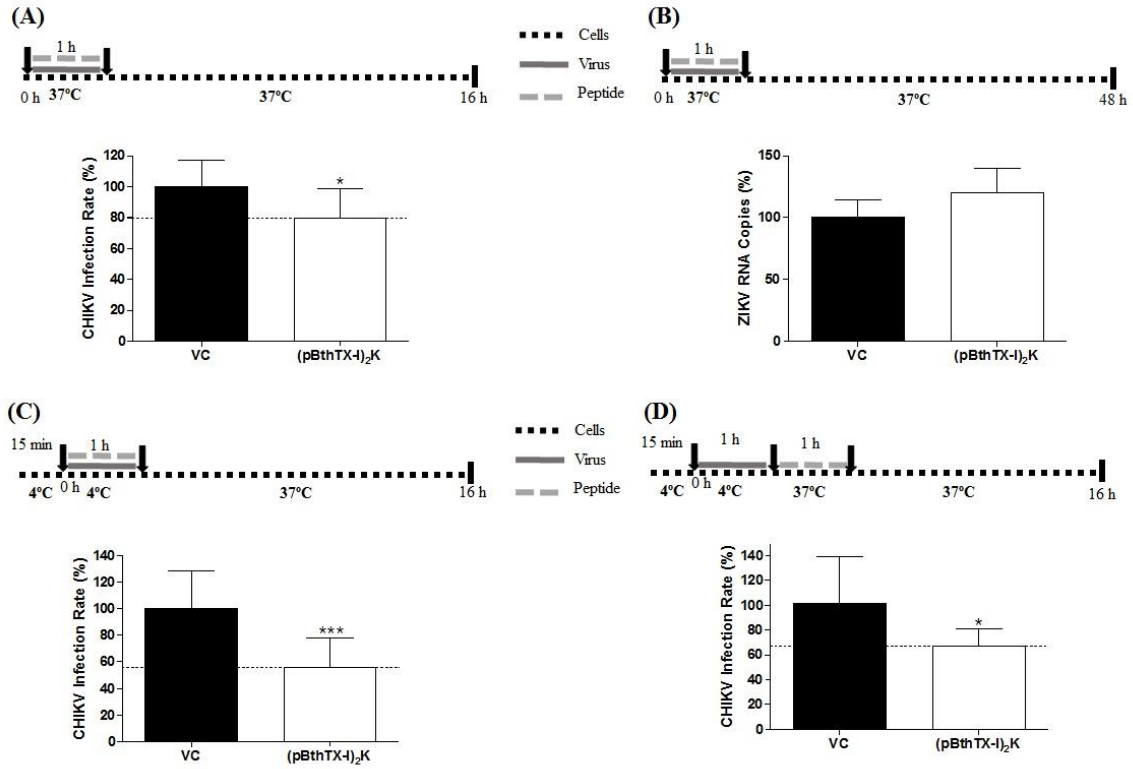
Virucidal Effect of the (p-BthTX-I)$_2$K Peptide on CHIKV and ZIKV Extracellular Particles

To determine whether the (p-BthTX-I)$_2$K peptide exerts a virucidal effect, CHIKV-NLuc or ZIKV virions were treated with the peptide at the MNTC for 1 hour and then used to infect the cells. In this assay, the (p-BthTX-I)$_2$K peptide did not demonstrate the ability to act on CHIKV and ZIKV virions, as indicated by failure to reduce the NLuc levels in CHIKV-NLuc-infected cells or in ZIKV RNA levels (Figures 2C and D).

(p-BthTX-I)$_2$K Peptide Inhibits the Early Stages of CHIKV Infection but Not the Early Stages of ZIKV Infection

The action of the (p-BthTX-I)$_2$K peptide on the early stages of CHIKV and ZIKV infection was evaluated. The peptide impaired CHIKV-NLuc entry into BHK-21 cells, resulting in a modest (20.3%) but significant ($p \leq 0.05$) reduction in viral replication levels in relation to those induced in the VC group (Figure 3A). In contrast to its effect on CHIKV infection, (p-BthTX-I)$_2$K did not inhibit the entry of ZIKV into cells (Figure 3B).

Figure 3. Effect of the (p-BthTX-I)$_2$K peptide on the early stages of CHIKV and ZIKV^{BR} infection. **(A)** The peptide treatment at 12.5 µM significantly inhibited CHIKV-NLuc (MOI 0.1) entry into BHK-21 cells. **(B)** The peptide treatment at 25 µM did not inhibit entry of ZIKV (MOI 0.1) into Vero cells. **(C)** (p-BthTX-I)$_2$K treatment at 12.5 µM exerted a significant inhibitory effect on CHIKV-NLuc attachment to BHK-21 cells. **(D)** The peptide treatment at 12.5 µM showed significant inhibitory activity on CHIKV-NLuc internalization into BHK-21 cells. CHIKV replication was analyzed via the measurement of the NLuc activity 16 h.p.i. The RNA levels of ZIKV were quantified by qRT–PCR 48 h.p.i. Error bars represent ± SD. Mean values ± SDs were calculated from the data obtained from a minimum of three independent experiments, each of which was performed in quadruplicate for the CHIKV assay and in duplicate for the ZIKV assay. ***:** $p \leq 0.05$; *****:** $p \leq 0.001$. **VC:** vehicle control (sterile water). A schematic representation of the respective experiment is shown above each graph.



(p-BthTX-I)$_2$K Peptide Affects Both the Cell Attachment and Internalization of CHIKV

The early stages of viral infection involve attachment and internalization of the virus in host cells [35]. Therefore, the antiviral effect of the (p-BthTX-I)$_2$K peptide on these specific steps of CHIKV entry was investigated to determine the behavior of this peptide in the early stages of viral infection.

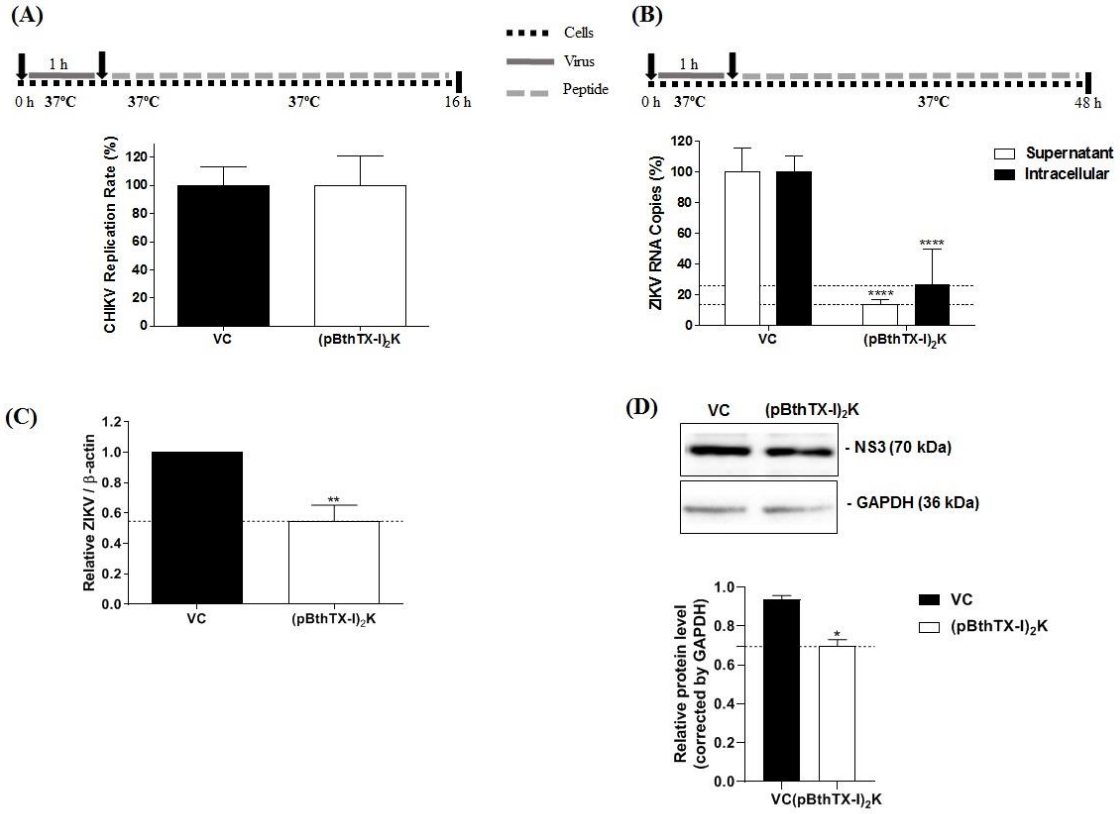
The (p-BthTX-I)$_2$K peptide significantly inhibited CHIKV entry by reducing viral attachment to BHK-21 cells by 44% ($p \leq 0.001$) compared with that induced by the VC (Figure 3C). Furthermore, (p-BthTX-I)$_2$K significantly inhibited CHIKV internalization. At this step, the CHIKV inhibition was 34.4% ($p \leq 0.05$) compared to that in the VC group (Figure 3D). The fact that these inhibitory effects were more prominent than those observed in the entry assay (Figure 3A) may reflect different experimental conditions (such as the use of a low-temperature step in the attachment and internalization assays).

(p-BthTX-I)$_2$K Peptide Inhibits the Postentry Steps of ZIKV but Not CHIKV Infection

Finally, we assessed the antiviral action of the (p-BthTX-I)$_2$K peptide on the postentry steps in the CHIKV and ZIKV infection cycles. The peptide did not exhibit an antiviral effect on the infection processes after CHIKV entry into BHK-21 cells (Figure 4A). In contrast, the peptide inhibited the postentry steps of ZIKV infection in Vero cells: a significant difference between peptide-treated and VC-treated cells was observed. The effect was also prominent: the level of ZIKV RNA in the supernatant was reduced by 86% ($p \leq 0.0001$), while the reduction in intracellular ZIKV RNA levels was 73.6% ($p \leq 0.0001$) (Figure 4B). qRT–PCR analysis using β-actin mRNA as the standard confirmed that in addition to the absolute levels of intracellular ZIKV RNAs, the relative levels (normalized to the mRNA of β-actin) were significantly ($p \leq 0.01$) reduced in the (p-BthTX-I)$_2$K-treated cells (Figure 4C). Considering the study of Batista and collaborators, we sought to verify whether the effect of the peptide on ZIKV postentry into cells may be related to the release step, and therefore, we calculated the difference in viral particles between intracellular and supernatant [28]. We observed that there was no significant reduction in viral RNA in the supernatant compared to viral RNA in the cells, indicating that (p-BthTX-I)$_2$K did not act on the release step.

To better investigate the postentry inhibition of ZIKV, we performed a Western blotting analysis to quantify the NS3 protein of this virus. The treatment of the cells after the infection by ZIKV with (p-BthTX-I)$_2$K reduced significantly the level of this protein ($p \leq 0.05$) compared with that in the cells treated with VC (Figure 4D).

Figure 4. Analysis of the effect of the (p-BthTX-I)$_2$K peptide on the postentry stages of CHIKV and ZIKV infection. **(A)** Effect of peptide treatment at 12.5 µM on the postentry steps of CHIKV-NLuc in BHK-21 cells. **(B)** Effect of the peptide treatment at 25 µM on the postentry stages of ZIKV in Vero cells as measured by the amount of virus RNA in the supernatant and infected cells. **(C)** Normalization of ZIKV RNA levels on the basis of the ratio of the total intracellular RNA to the mRNA of β-actin. CHIKV replication was analyzed via the measurement of NLuc activity 16 h.p.i. The levels of ZIKV^{BR} RNA were quantified by qRT–PCR 48 h.p.i. **(D)** NS3 ZIKV protein expression in the postentry assay. The level of this protein was quantified using western blot analysis. Error bars represent ± SD. The mean values ± SDs represent data from a minimum of three independent experiments that were each performed in quadruplicate for the CHIKV assay and in duplicate for the ZIKV assay. ***:** $p \leq 0.05$; ****:** $p \leq 0.01$; ******:** $p \leq 0.0001$. **VC:** vehicle control (sterile water). A schematic representation of the respective experiment is shown above each graph.



Discussion

The CHIKV and ZIKV arboviruses represent an emerging health threat. Although the mortality of diseases caused by these viruses is considered to be low, the morbidity associated with CHIKV infection is high, causing social and economic impacts [36]. ZIKV infection is also a concern due to neurological alterations, such as congenital Zika syndrome and Guillain–Barré syndrome [37]. Since no antivirals or vaccines have been approved prevent or treat these viruses, it is necessary to search for potential therapeutics [11,17].

Peptides exhibit characteristics that favor their use in therapeutic approaches, such as cellular biocompatibility and low toxicity [18-20]. The peptide (p-BthTX-I)$_2$K is a synthetic peptide with the structure based on another peptide [(p-BthTX-I)$_2$] derived from the BthTX-I toxin in snake venom, which had previously demonstrated activity against several Gram-positive and Gram-negative bacteria [23,38] and antiviral activity against SARS-CoV-2 [24], which is an enveloped positive-sense single-stranded RNA virus similar to ZIKV and CHIKV. In this study, we assessed the antiviral activity of the (p-BthTX-I)$_2$K peptide against CHIKV and ZIKV infection, as well as the steps in the viral replicative cycle in which this peptide shows activity. Our results demonstrated that this peptide reduced the infection of these viruses in a dose-dependent manner, with the antiviral effect against ZIKV being more pronounced than that against CHIKV. Notably, antiviral compounds can act at different stages of the replication cycle [39,40]. Somewhat unexpectedly, we found that the steps of the infection inhibited by (p-BthTX-I)$_2$K peptide were completely different for these viruses. The peptide inhibited CHIKV infection by interfering with the early steps of the viral replication cycle, preventing CHIKV entry into BHK-21 cells. In contrast, (p-BthTX-I)$_2$K pretreatment protected cells against ZIKV, and the peptide also inhibited the postentry steps of ZIKV infection.

The entry stage of CHIKV into host cells is divided into two steps. First, viral particles attach to the cell surface. The nonspecific interaction between cell and virus is followed by specific binding between the E2 glycoprotein present on the surface of viral particles and specific receptors on host cells, such as the cell adhesion molecule Mxra8, the main receptor of CHIKV, as well as the mucin-1 (TIM-1) protein, type 1 prohibition (PHB1) protein and glycosaminoglycans (GAGs). This interaction triggers CHIKV internalization into cells through clathrin-mediated endocytosis. After endosome acidification, the E1 glycoprotein in the viral envelope undergoes a conformational change, resulting in the exposure of the hydrophobic fusion peptide and subsequent fusion of viral and endosomal membranes. After this process, the nucleocapsid is released into the cell cytoplasm and is disassembled, enabling

the release of viral RNA [35,41]. Thus, the viral entry process involves different factors, making this step a target for several antiviral compounds [42].

We have shown that the (p-BthTX-I)$_2$K peptide inhibited CHIKV entry by interfering with both attachment and internalization of the virus into BHK-21 cells. Furthermore, we observed that viral attachment was affected to a greater extent than viral internalization into these cells. We also demonstrated that the peptide did not exert an inhibitory action on CHIKV entry into BHK-21 cells by protecting the cells against viral infection or by damaging the viral particle, since this peptide did not exhibit a virucidal effect and because pretreatment of the cells provided no protection against subsequent CHIKV infection. It is not easy to explain these findings, as most compounds with an inhibitory effect on viral entry act through two main mechanisms: (1) direct action on the viral structure, inactivating virions and thus preventing their interaction with the host cell and/or (2) interference with host cell components that are crucial to viral infection [27,43]. In summary, our data showed that the (p-BthTX-I)$_2$K peptide exerts its effect against CHIKV infection as a cotreatment. The inhibitory effect on viral attachment suggests that one of the antiviral modes of action for this peptide is interference of the binding of the viral glycoprotein E2 with cell receptors. One of the characteristics of peptides that favors their therapeutic use is cellular biocompatibility. Peptides are natural ligands for several cell surface receptors, demonstrating high affinity and specificity [18-20]. In addition, the (p-BthTX-I)$_2$K peptide is a cell-penetrating peptide (CPP) [21]. Peptide in this class enter eukaryotic cells without damaging the plasma membrane. Neundorf (2019) reported that GAGs, which have been indicated to be cellular factors important for CHIKV binding, are essential for the interaction of CPPs with cells [44]. Therefore, as CPPs and CHIKV share cell receptors, competition between peptides and viruses for binding sites on the cells may explain the peptide antiviral effects. Most likely, the (p-BthTX-I)$_2$K peptide shows greater affinity than the E2 viral glycoprotein for cell receptors, such as GAGs, reducing the number of viral particles that can enter cells.

The antiviral compounds that prevent CHIKV internalization in host cells mainly target clathrin-mediated endocytosis and endosome acidification [42]. As the main entry mechanism of CPPs into eukaryotic cells is endocytosis [44], we hypothesized that when added concomitantly to BHK-21 cells, the virus and (p-BthTX-I)$_2$K peptide internalize into cells via the same endosome. Therefore, the (p-BthTX-I)$_2$K peptide can exert its antiviral effects on CHIKV internalization by (1) blocking the reduction in pH in the endosomal environment, preventing the conformational change of the E1 viral glycoprotein and blocking the subsequent fusion of the viral and endosomal membranes and/or (2) interacting with the E1 viral glycoprotein, preventing the exposure of the hydrophobic fusion peptide and subsequent fusion of the viral

envelope and cell membrane [39]. Finally, it is plausible that CPPs cause premature or delayed release of the virus genome into the cytoplasm, which may hamper subsequent viral infection steps.

Our work also demonstrated that the (p-BthTX-I)$_2$K peptide did not cause an antiviral effect on the later stages of the CHIKV replication cycle. Therefore, this peptide did not affect the steps that follow CHIKV entry into BHK-21 cells, such as the synthesis of viral RNA, production of viral nonstructural and structural proteins, or assembly and release of viral particles [39].

Moreover, the (p-BthTX-I)$_2$K peptide showed a protective effect against ZIKV infection in Vero cells but did not act on the viral entry step. Similar to CHIKV, the GAGs required for the interaction of CPPs with cells may be attachment factors for ZIKV entry into cells. However, although ZIKV and CPPs may share attachment factors [44,45], the impact of (p-BthTX-I)$_2$K treatment was clearly different in CHIKV and ZIKV infection contexts. Protection against ZIKV infection required peptide pretreatment of cells; i.e., the peptide needed to be present before exposure to a virus. In the cotreatment case, the peptide was unable to inhibit viral entry, and ZIKV seemed to compete more successfully for binding cell receptors. In addition, considering that endocytosis is the main mechanism by which CPPs enter cells [44], we hypothesized that after with (p-BthTX-I)$_2$K pretreatment of Vero cells, the peptide was internalized by endocytosis, loaded at ZIKV postentry sites in cells and acting during ZIKV postinfection of cells. If this hypothesis is supported, the protective effect of pretreatment may be related to the mechanism used for the inhibition of ZIKV postentry infection.

The postentry stages of positive-strand RNA virus infection include genome translation, RNA replication, and virion assembly and release [46]. In summary, after the cell entry and internalization steps, ZIKV RNA is released into the cytoplasm and translated into viral proteins from the only open reading frame (ORF) in the viral RNA; the translation product is a polyprotein that is cleaved into structural and nonstructural proteins. RNA replicase is formed by nonstructural proteins associated with intracellular membranes [47,48] and binds viral RNA to initiate RNA replication. First, negative-sense RNA is synthesized, and then, new positive-sense single-stranded RNAs are synthesized. Next, the new viral RNAs are packaged by structural proteins, forming immature enveloped virions budding into the endoplasmic reticulum. These virions move through the secretory pathway in the trans-Golgi network, maturing after the cleavage of the prM protein to form the M protein, and the new virions are then released from the cell by exocytosis [47,48].

A significant reduction in RNA level in the intracellular and extracellular environment of treated Vero cells may indicate that (p-BthTX-I)$_2$K acts on the RNA replication steps [49]. Freire

and collaborators verified that (p-BthTX-I)$_2$K showed inhibitory potential against the protease PL^{pro} activity of SARS-CoV-2, and the authors suggested that peptides derived from BthTX- I, such as (p-BthTX-I)$_2$K, may be useful for inhibiting the enzymatic activities of viral and cellular proteins [24]. The inhibition of these activities may be related to their effects on ZIKV postentry steps. SARS-CoV-2 is a positive-sense single-stranded RNA which functions as mRNA that translates viral polyproteins, and viral proteases cleave these polyproteins to generate viral proteins [50], and ZIKV follows a similar process. The potential of (p-BthTX-I)$_2$K to inhibit enzymatic activities, as observed with SARS-CoV-2, may be related to its effect on the postentry steps of ZIKV infection, with the potential target being the NS2B-NS3 complex (protease) or NS5 (RNA polymerase) of ZIKV [46].

In fact, the Western blotting analysis showed a reduction in NS3 protein in treated cells, so this may be interfering with the NS2B-NS3 complex. Furthermore, the NS3 protein of ZIKV has other important rules in replication. The C-terminal domain of this protein is a helicase, which acts in the RNA synthesis unwinding the dsRNA [48,51]. This domain also has nucleoside 5'-triphosphatase, which is necessary to format the cap of the 5-terminal of the RNA [48]. The NS3 inhibition may indicate that the (p-BthTX-I)$_2$K interferes with ZIKV replication.

However, the effect may also be indirect; for example, flavivirus RNA is replicated on the membranes of the endoplasmic reticulum, and (p-BthTX-I)$_2$K alteration of the structure/composition/properties of this cellular compartment would exert a negative impact on ZIKV RNA replication as well. Other possible explanations include the suggestion that the peptide interferes with the viral assembly and/or maturation of virions. Our results showed that viral release did not seem to be affected by (p-BthTX-I)$_2$K, as the peptide causes a similar reduction in extracellular and intracellular RNA levels. However, more studies are needed to clarify the effect of (p-BthTX-I)$_2$K on the ZIKV replication cycle.

In conclusion, the data showed from our study that the (p-BthTX-I)$_2$K peptide shows antiviral activity against CHIKV infection by interfering in the early stages of viral infection. The peptide inhibited viral entry into BHK-21 cells by impairing attachment and internalization into the host cell. In addition, our study demonstrated that the inhibitory action of the (p-BthTX-I)$_2$K peptide on CHIKV entry was more significant on viral attachment than on internalization of the virus in BHK-21 cells. For ZIKV, this peptide caused a protective effect in Vero cells and decreased the ZIKV RNA level by inhibiting postentry steps of the viral infection cycle. Therefore, this work suggests that the (p-BthTX-I)$_2$K peptide may be a promising antiviral drug candidate against CHIKV and ZIKV, and based on its antiviral properties, suggests the use of

this peptide as a template structure in the development of new broad-spectrum antiviral derivatives.

Supplementary Materials

The following supporting information can be downloaded at: https: //www.mdpi.com/article/10.3390/v15051168/s1. 1. Determination of the maximum non-toxic concentration (MNTC) in BHK-21 and Vero CCL-81 cells; Figure S1. Cytotoxic effect of the (pBthTXI)2K peptide in (A) BHK-21 cells and (B) Vero cells. Black lines represent the arbitrary limit of 80% cell viability. VC: vehicle control (sterile water). Error bars represents ± standard deviation (SD).

Author Contributions

Conceptualization: G.M.A., M.L.D.L., P.R.d.S.S., C.B., M.d.F.C. and P.R.; Methodology: G.M.A., M.L.D.L., P.R.d.S.S., I.A.S., D.O.S.M., P.J.P.d.C., T.C., V.G.d.C., C.B., N.A.S.-F., E.M.C., A.C.G.J., M.d.F.C. and P.R.; Formal Analysis: G.M.A., M.L.D.L. and P.R.d.S.S.; Investigation: G.M.A., M.L.D.L. and P.R.d.S.S.; Resources: A.M., E.M.C., A.C.G.J. and P.R.; Writing—Original Draft Preparation: G.M.A. and M.L.D.L.; Writing—Review and Editing: P.R.d.S.S., I.A.S., V.G.d.C., C.B., A.M., E.M.C., A.C.G.J., M.d.F.C. and P.R.; Supervision: C.B., E.M.C., M.d.F.C. and P.R.; Project Administration: M.d.F.C. and P.R.; Funding Acquisition: G.M.A., M.L.D.L., E.M.C., M.d.F.C. and P.R. All authors have read and agreed to the published version of the manuscript.

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Not applicable.

Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary Material

The Dimeric Peptide $(KKYRYHLKPF)_2K$ Shows Broad-Spectrum Antiviral Activity by Inhibiting Different Steps of Chikungunya and Zika Virus Infection

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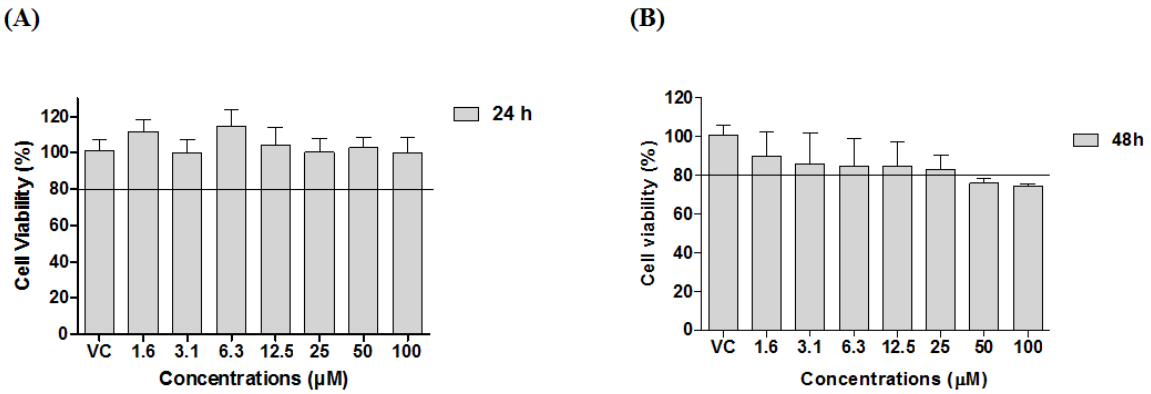
1. Determination of the maximum nontoxic concentration (MNTC) in BHK-21 and Vero CCL-81 cells

The cytotoxicity of the $(pBthTX\text{-}I)_2K$ peptide was analyzed in BHK-21 cells at 24 hours or 48 hours in Vero cells by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The peptide was tested at concentrations of 1.6, 3.1, 6.3, 12.5, 25, 50 and 100 µM in BHK-21 and Vero cells. The maximum nontoxic concentration (MNTC), expressed in µM, was defined as the highest concentration of the peptide that maintained at least 80% of the cells viable within 24 hours or 48 hours. Considering the arbitrary threshold of 80% cell viability, the $(pBthTX\text{-}I)_2K$ peptide reached its MNTC in BHK-21 cells at 12.5 µM at 24 hours (Figure S1A) and 25 µM in Vero cells at 48 hours (Figure S1B).

Figure S1. Cytotoxic effect of the $(pBthTX\text{-}I)_2K$ peptide in **(A)** BHK-21 cells and **(B)** Vero cells. Black lines represent the arbitrary limit of 80% cell viability. **VC:** vehicle control (sterile water). Error bars represents ± standard deviation (SD).



Capítulo III

1. CONCLUSÕES

Nossos dados representaram os primeiros relatos de atividade antiviral do peptídeo (p-BthTX-I)$_2$K e do protótipo peptídico AG-Hecate e seus análogos PSSct1905 e PSSct1910 contra a infecção por CHIKV. Dentre todos os peptídeos testados, o AG-Hecate e seu análogo PSSct1905 foram os mais ativos. O peptídeo (p-BthTX-I)$_2$K apresentou ação antiviral ao interferir nas etapas iniciais do ciclo de replicação viral. O mesmo impediu a entrada do CHIKV em células BHK-21 por meio da inibição das etapas de adsorção e internalização viral. Além disso, nosso estudo demonstrou que o efeito inibitório do peptídeo (p-BthTX-I)$_2$K na entrada viral foi mais significativa na adsorção do que na internalização do CHIKV em células BHK-21.

O protótipo peptídico AG-Hecate e seus análogos, por sua vez, interferiram em diferentes etapas do ciclo de infecção do CHIKV em células BHK-21 e Huh-7. O análogo PSSct1905 exibiu efeito protetivo nas células contra a infecção viral. A entrada do CHIKV em ambas as linhagens celulares foi afetada pelos peptídeos PSSct1905 e PSSct1910, desencadeando inibição da adsorção e internalização viral. Por fim, diferentemente do peptídeo (p-BthTX-I)$_2$K, o protótipo AG-Hecate e seus dois análogos apresentaram atividade antiviral nas etapas de pós-entrada do ciclo de replicação viral nas duas células avaliadas. Portanto, esse trabalho potencializa o peptídeo (p-BthTX-I)$_2$K e o protótipo peptídico AG-Hecate e seus análogos PSSct1905 e PSSct1910 como promissores candidatos a fármaco antiviral contra o CHIKV. Ademais, considerando as propriedades antivirais desses peptídeos, sugere-se a utilização da estrutura dos mesmos como base para o planejamento de novos antivirais de amplo espectro.

Capítulo IV

1. PATENTE

CARNEIRO, B. M.; BITTAR, C. O.; CILLI, E. M.; **AYUSSO, G. M.**; LIMA, M. L. D.; BATISTA, M. N.; CALMON, M. F.; RAHAL, P.; SANCHES, P. R. S.

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2. ARTIGO CIENTÍFICO

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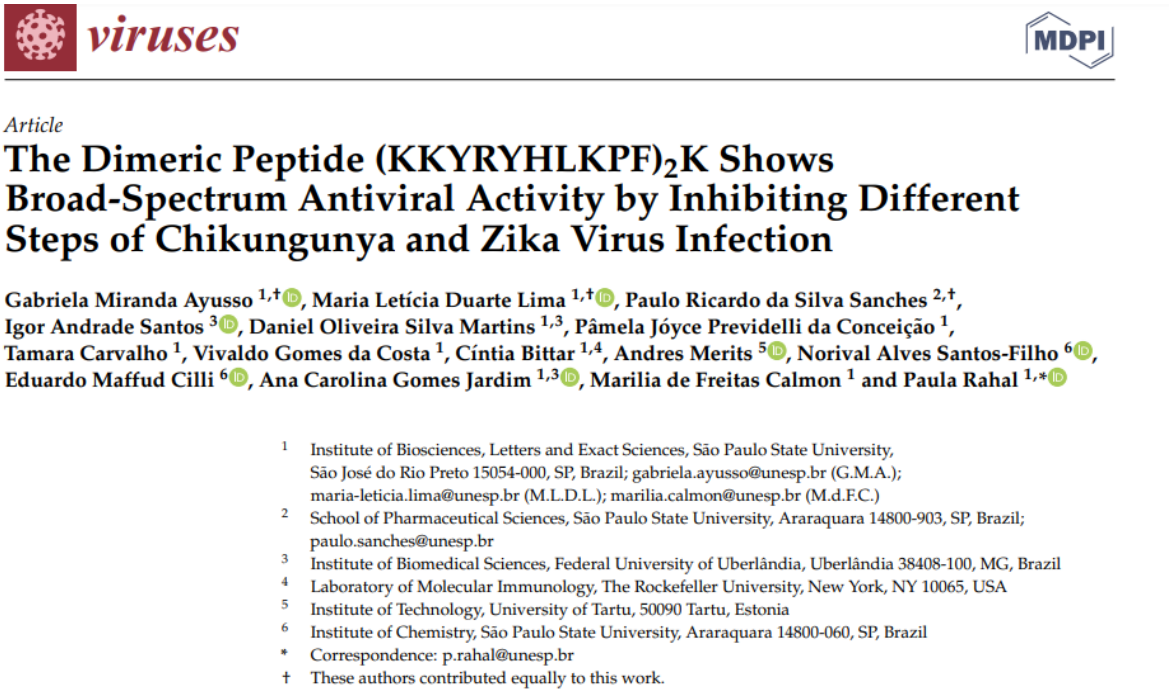

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Article

The Dimeric Peptide $(KKYRYHLKPF)_2K$ Shows Broad-Spectrum Antiviral Activity by Inhibiting Different Steps of Chikungunya and Zika Virus Infection

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